HINDUSTAN ANTIBIOTICS

Bulletin

NOVEMBER 1960

VOL. 3

2

HINDUSTAN ANTIBIOTICS

Bulletin

Vol. 3 November 1960 No. 2

EDITORIAL & ADVERTISEMENT OFFICES

Hindustan Antibiotics Ltd., Pimpri, Poona Dist.

This *Bulletin* is published on the 15th of February, May, August & November, and contains articles and reviews on all aspects of antibiotics production and use.

The views expressed in this journal are those of the authors and do not necessarily represent those of the Company or of the editors.

Annual subscription : Rs. 3.00 (inland), 8 sh. or $1.50 (foreign). Single copy: Re. 1.00 (inland), 2sh. 6d. or $0.50 (foreign).

CONTENTS

ADVERTISERS INDEX

We are proud of the reputation we have earned for the first class quality of our products, the fairness of our dealings and our wide distribution and, in this manner, of our service, to India.

GEOFFREY MANNERS & CO. PRIVATE LTD.

Manufacturers of

Anacin • Kolynos • Bismag • Anne French • Aludrox • Endrine • Forhan's • Manners Cough Syrup • Mink Pain Balm • Manners Gripe Mixture • Vaseline Hair Tonic • Wyeth Ethical Products • Manners Ethical Products.

K. 47 (a)

USE OF ANTIBIOTICS IN FOOD PRESERVATION

Spoilage of perishable food-stuffs due to microorganisms is a major problem in food technology, especially in underdeveloped and tropical countries where adequate refrigeration facilities are not available. Food preservation methods in use even to-day include drying, smoking, salting, fermentation, canning, etc. These conventional methods, however, have their shortcomings particularly in changing flavour and taste. Freezing preserves the freshness of the food, but the method is expensive and not applicable to many types of perishable food materials.

The importance of antibiotics as chemical preservatives in the food industry was recognized following the discovery that broad-spectrum antibiotics like the tetracyclines inhibit the growth and production of enzymes by bacterial populations. Commercial application of chlortetracycline in food industry in the United States of America became possible only in 1955, when the Food and Drug Administration permitted such use, with residue 5-7 p. p. m. Of the several antibiotics including penicillin, streptomycin, neomycin, polymyxin, tetracyclines, subtilin, and nisin, tested in food industry for various purposes, only the last three have proved to be of use. The antibiotics, though active in low concentrations, are quickly inactivated in aqueous media. They are mainly useful in conjunction with other conventional preservation methods in prolonging the "shelf-life" of perishable food stuff either during transit or storage period before processing.

Canning food materials is known to impart adverse taste to the product due to the high temperatures used in sterilization. In 1950, Anderson and Michner reported that the combined action of the antibiotic subtilin and mild heat could preserve food-stuffs. It was later shown that several of the toxin producing spore-formers like *Clostridium botulinum* are not completely eliminated, and there is no short-cut to complete heat sterilization. The antibiotic nisin produced by *Streptococcus lactis*, a natural inhabitant of dairy products has been shown to be useful in preventing Clostridial spoilage, and is preferable to subtilin.

The preservation and delaying spoilage of perishable fresh foods such as fish, meat, poultry, milk, vegetables and fruits, with antibiotics as adjunct to refrigeration has been worked out on commercial scale. Storage of chopped fish in ice containing 5 p. p. m. chlortetracycline keeps them in a fresh state for 8 to 13 days. Administration of chlor- and oxytetracycline to poultry and cattle before slaughter prolongs the storage period of carcasses and prevents their infestation by dangerous bacterial forms. This processing has been further helpful in tenderizing steaks. Spraying of slaughtered animals with tetracyclines helps storage under conditions where refrigeration is inadequate.

Fruits, vegetables and dairy products are subject to spoilage during transit, both by fungi and bacteria. Dip treatments with streptomycin or tetracyclines along with nystatin could considerably increase the storage period of vegetables. Addition of only 1 p. p.m. of tetracycline to raw milk directly after milking, is known to enhance the storage period by 24 hours at 37°C. Antibiotics have been successfully used in the preservation and in enhancing the storage period of several other food products.

Continued use of antibiotics in food preservation brings in the problem of cumulative antibiotic residues and hence poses a public health problem. In the concentrations recommended, however, there appears to be no adverse effect directly on human beings, nor does it induce development of drug resistant strains of pathogens. The future prospects in preservation of perishable food-stuffs appear to be a combination of antibiotics with irradiation and refrigeration.

The Polyene Antifungal Antibiotics

L. C. VINING
National Research Council of Canada, Prairie Regional Laboratory, Saskatoon, Saskatchewan.

Introduction

Since the discovery of streptomycin from a culture of *Streptomyces griseus* in 1944, by Waksman and co-workers, screening programmes for new and useful antibiotics have devoted increasing attention to the actinomycetes. One result has been a plethora of partially characterized products which for many years has disheartened the conscientious reviewer and discouraged attempts at classification. However, growing interest in the chemical structure of these metabolic products has recently revealed a number of broad natural classes of compounds and a clearer picture of the biosynthetic pathways operating in this hitherto neglected order of microorganisms. One of the largest such groups was first recognized[1] by the characteristic type of light absorption spectrum resulting from the presence in the molecule of a series of conjugated, unsaturated double bonds[2] and the members are now generally referred to as "polyene" antibiotics. They also have in common high activity against a wide variety of yeasts and fungi but little or no effect on bacteria, are all somewhat unstable compounds, particularly in light or in weakly acidic or alkaline solution, and show a marked lack of solubility in non-polar solvents or water at neutral pH. Other characteristic physical or chemical properties have been less easily discernible, partly because, as a result of difficulties in purification, a large number of substances have been described only as crude preparations and named as new antibiotics on the basis of differences in biological activity. In addition, the stronger interest in this latter aspect of a new product has often led to neglect in describing elementary chemical and physical properties and until recently to lack of information on their chemical structure.

Isolation

Aqueous alcohols have been found the most suitable agents for extracting polyene antibiotics. Where the activity resides exclusively in the mycelium or culture filtrate, it usually can be efficiently extracted with methanol or *n*-butanol, respectively, but when it is necessary to recover the product from both broth and solids it has been found convenient to mix the whole culture with a water-miscible alcohol and recover the activity from the filtrate. Upon concentration at neutral pH, the polyene antibiotic is precipitated, often in a fairly pure condition.

The ease with which members of the group may be obtained completely pure appears to vary. Polyene antibiotic-producing cultures frequently also produce substances capable of dispersing the water-insoluble antibiotic in the broth. Variations in the amounts of such solubilizers produced by different cultures are reflected in the irregular distribution of polyene antibiotics between broth and mycelium.[3] Active material from low yielding cultures is often contaminated with overwhelming amounts of such impurities and cannot be purified by solvent fractionation.[4] Resort to more exacting methods, e.g. chromatography or countercurrent distribution, may be successful, but the amounts obtained in

this way are usually limited by poor solubility. After some degree of purification has been achieved, the problem of removing the remaining contaminants may also be complicated by the low solubility of the product, particularly where the use of solvents or conditions suitable for dissolving the sample leads to rapid loss of potency. In general, the solubility of the polyenes is poor in low boiling esters, alcohols, and ketones but is improved appreciably by the addition of up to 30% water. Certain members of the group (e.g. filipin or fungichromin) are soluble enough to be purified by recrystallization from these solvents. Others (e.g. amphotericin B or candidin) are almost insoluble and are best dissolved by the use of dimethyl formamide, dimethyl sulfoxide or pyridine. Hosoya and Hamamura[5] have described a mixture consisting of pyridine - dioxane - *n* - butyl acetate-water (30:40:18:52) suitable for dissolving large quantities of crude trichomycin and from which the antibiotic is precipitated in good yield on cooling. A number of these antibiotics form complexes with calcium chloride which are fairly soluble in methanol and may be conveniently used in their purification.[6]

Properties

More than thirty polyene antibiotics have now been obtained in a crystalline state and/or sufficiently well characterized to indicate that they are probably different from other known compounds. Variations in the wave lengths at which their characteristic light absorption maxima occur can be used to separate them into four major sub-groups which correspond with the presence in the antibiotic of a chain of 4, 5, 6 or 7 conjugated double bonds.[2] Obviously a large number of structural variations in the remainder of the molecule must be possible and account for the differences in stability and solubility noted above, and also for differences in biological activity. Variations in relative activity against susceptible microorganisms are accompanied by different degrees of toxicity in animals, and only three members, nystatin, amphotericin B and trichomycin, from this large family of related compounds have been found suitable for clinical use. Although not ideal chemotherapeutic agents because of their poor solubility and moderate toxicity, their high specific activity against pathogenic yeasts and fungi has afforded them a useful role in the treatment of diseases which are otherwise inaccessible to drug therapy.

Studies on mode of action of several polyene antibiotics have indicated common aspects but considerable work remains to be done before it can be established whether all inhibit by the same mechanism and what factors are responsible for the differences in their activity. Work on inhibition by nystatin[7,8] has revealed a specific effect on substrate utilization and demonstrated a relationship between binding of the antibiotic and its activity. The activity of many polyene antibiotics has been shown[9,10] to be reversed by certain sterols, an observation which might indicate[10] that they interfere with an essential role of sterols in the metabolism of yeasts and fungi. Continuation of such studies should eventually explain why bacteria are unaffected by the polyenes, and incidentally increase our knowledge of the metabolism and structure of fungi as much as that of bacteria has been increased by similar work with antibacterial antibiotics.

A summary of the properties of many of these antibiotics has already been given by Dutcher[11] and more recently their physico-chemical properties have been tabulated.[12] However, for convenience some of the important physical and chemical properties of the named polyenes are listed again in Tables I to IV and the individual compounds are discussed briefly in the following paragraphs.

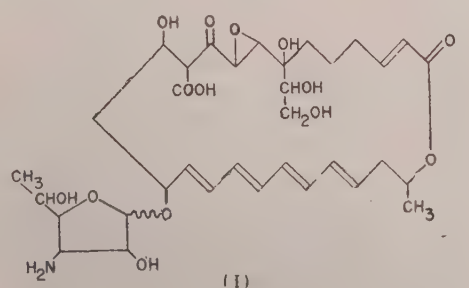
Tetraenes

Nystatin, originally reported under the name 'fungicidin' and the first polyene

antibiotic to be isolated, was obtained in 1950 from a culture of *Streptomyces noursei*[13] which simultaneously produced cycloheximide. The preparation of pure nystatin and a description of chemical and physical characteristics were not announced until several years later,[14] and during this interval three similar antibiotics were reported. Rimocidin, an antifungal agent isolated from *Streptomyces rimosus* and purified as the crystalline acid or basic salt,[15] showed a striking resemblance to nystatin in solubility and ultraviolet absorption spectrum, but could be distinguished from it by differences in optical rotation, elementary analysis, infrared spectrum and the stability of its salts. A strain of *Streptomyces chromogenes* yielded another crystalline antibiotic, chromin,[16] which again resembled nystatin, but was not identical with it. Chromin and rimocidin proved to be even more closely related,[17] but differences in the published analytical and spectral data suggest that they are not the same substance. The third tetraene was obtained as an impure preparation from *Streptomyces aureus*.[18] The similarity of its ultraviolet absorption spectrum to that of nystatin was noted and although it was not claimed as a new compound, its biological activity showed differences from other known antibiotics. Subsequent work[19] has demonstrated that the activity of the original product was due to a mixture of related compounds, one of which (antimycoin A) is paper-chromatographically distinguishable from nystatin, but not from rimocidin or chromin. No further information has been reported which might indicate whether it is, in fact, identical with either of the latter two antibiotics. One other tetraene, sistomycosin, is a contemporary of these products, although a description of its properties did not appear in the patent literature until 1955.[20] It was isolated from *Streptomyces viridosporus*, but does not appear to have been obtained pure. No published information on its relationship to other tetraenes is available.

Amphotericin A is the only tetraene yet isolated in a pure state from a culture producing polyene antibiotics with mixed chromophoric systems. *Streptomyces nodosus*,[21] also produces the heptaene, amphotericin B, but the two antibiotics can be separated by the solubility of the tetraene in methanolic calcium chloride solution. Amphotericin A has been reported to differ from nystatin and rimocidin[22] and is paper-chromatographically distinguishable from chromin and antimycoin A.[19] Although other *Streptomyces* cultures producing tetraenes simultaneously with heptaenes,[3] pentaenes[3] and hexaenes [1, 4, 23] respectively, have been reported, none has yet been described in sufficient detail to establish its relationship to known tetraenes. An antibiotic designated 7-071 R. P., isolated from a *Streptomyces* species which also produced two antibacterial compounds, has been shown to be a tetraene[25] and reported to have an infrared spectrum distinct from those of nystatin, rimocidin and chromin.

Pimaricin, first isolated by a group in Holland[26] from *Streptomyces natalensis*, has been distinguished from nystatin, rimocidin and amphotericin A by its mobility in various solvent systems, and its paper-chromatographic behaviour indicates that it is probably different also from chromin and the antimycoins. It was isolated independently at the Lederle Laboratories in the United States, and an intensive study of its chemistry by this group[27] has resulted in the proposed structure (I) for



(I)

TABLE I.—The tetraenes

§ Estimated from published spectra or calculated from published data.

Abbreviations: 2,4—DNPH —2:4 dinitrophenylhydrazine
DMF – dimethylformamide
DMAC —dimethylacetamide

Antibiotic	m.p.	Molecular composition	$\lambda_{max.}$ in mμ ($E^{1\%}_{1\,cm}$)	I.R. max. (μ) C=O str. region	(∝) D	Ionizable groups	Colour reactions and functional group tests
Nystatin	d.>160°	C, 58.42; H, 8.18; N, 1.6%; 2 C.CH_3 $C_{46}H_{77}NO_{19}$	292 §(560) 304.5 (850) EtOH 318 §(770)	5.87 6.37	—8° (HOAc) +21° (pyridine) +12° (DMF) —7° (acid MeOH)	Amphoteric	Red-brown with conc. H_2SO_4; pos. Molisch (faint), Schiff (slow); neg. Tollens, Fehling, 2, 4-DNPH
Rimocidin	151° (d.) (sulphate)	§C, 61.2; H, 8.2; N, 1.9% $C_{37}H_{59}NO_{13}$	291 §(620) 80% 304 §(980) MeOH 318 §(900)	5.86 6.12 6.51	+75° (acid MeOH)	Amphoteric	Red-brown with conc. H_2SO_4
Chromin	145-150°(d.)	C, 58.19; H, 7.81; N, 2.29%	292.5 305 EtOH 320	5.9 6.4	—	Acidic	Pos. Fehling; doubtful Tollens; neg. ninhydrin, $FeCl_3$, Molisch, Sakaguchi.
Antimycoin	—	—	291 304-5 EtOH 318	—	—	—	—
Sistomycosin	d. ca 130°	—	292.5 (250) 306 (340) H_2O 320.5 (295)	6.34	—	—	Cherry red-chocolate in conc. H_2SO_4; pos. Benedict, Molisch; neg. $FeCl_3$
Amphotericin A	d. >153°	C, 60.32; H, 8.39; N, 1.72% §$C_{44}H_{75}NO_{17}$	291 §(520) 304 §780) MeOH 318 §(715)	§5.8 6.4	+93° (HOAc) +163°(pyridine) +136°(DMF) +28° (acid MeOH)	Amphoteric	Pos. Molisch; neg. $FeCl_3$.
7·071 RP	275-280° (d)	C, 58.3; H, 8.0; N, 1.65%	291 (562) 304 (863) 318 (783)	—	+90° (MeOH) +80° (pyridine)	—	—
Pimaricin	d. ca 200°	C, 58.59; H, 7.32; N, 2.01%; 2 C. CH_3 $C_{34}H_{49}NO_{14}$	290 §(710) 303 §(1100) MeOH 318 §(1020)	5.84 §6.35	—	Amphoteric	Wine-red with conc. H_2SO_4; pos. Van Slyke N, iodoform.
PA 166	—	C, 59.59; H, 7.66; N, 2.00%; 3 C.CH_3 $C_{35}H_{53}NO_{14}$	291 §(715) 304 (1098) 80% 319 §(990) MeOH	§5.8 6.4	+275°(pyridine) +257°(DMF) +191°(acid DMF)	Amphoteric	Violet with conc. H_2SO_4; pos. ninhydrin, Fehling, 2, 4-DNPH.

Etruscomycin	150° (d.)	C, 59.57; H, 8.05; N, 2.04% $C_{36}H_{57}NO_{14}$	§290 (840) 305 (1390) §319 (1180)	5.86 6.35	+296°(pyridine) +50° (acid MeOH)	Amphoteric	Red-brown in conc. H_2SO_4; pos. Molisch (brown); neg. $FeCl_3$.
Unamycin A	148-150°(d.)	C, 52.24; H, 7.77; N, 1.74%	290 (643) 304 (1010) MeOH 319 (875)	5.9	—92°(80% MeOH) +79°(DMF)	Acidic	Red-brown →black in conc. H_2SO_4; pink. with Schiffs reagent; pos. Molisch; neg. Millon, Fehling, Tollens
Toyokamycin	—	—	—	—	—203°(MeOH) +216°(DMF)	—	—
Protocidine	d. 120°	—	290 (700) 80% MeOH 303 318	—	—	Amphoteric	Black-brown in conc. H_2SO_4; green with Fehling's solution; neg. $FeCl_3$, Molisch, ninhydrin, anthrone.
Akitamycin	—	C, 57.26; H, 7.68; N, 1.64%	291 303.5 319	—	+88° (80% MeOH) +158°(DMF)	—	—

pimaricin. A tetraene recently isolated from *Streptomyces chattanoogensis* and named tennecetin[28] has subsequently been discovered[29] to be identical with pimaricin.

Mycelial extracts of another *Streptomyces* species have afforded a tetraene designated PA 166[30] which has been purified and shown to differ chemically and biologically from all other well characterized antibiotics of this type. Etruscomycin[31] from *Streptomyces lucensis* resembles PA 166 in composition, infrared spectrum and optical rotation but appears to differ in the more intense absorption of the chromophore. The molecular extinction of etruscomycin (101,400), higher than other tetraene antibiotics and all-trans unsubstituted conjugated tetraenes (ca. 80,000), is surprising. Unamycin A,[32] a crystalline antifungal antibiotic recently isolated from strain U-10 of *Streptomyces fungicidicus*, may be distinguished from other tetraenes by its lack of basic properties, despite the presence of nitrogen in the molecule. A second antifungal substance, unamycin B, which resembles seligocidin, is produced simultaneously by the organism. The relationship between unamycin A and the tetraenes obtained from another strain of *Streptomyces fungicidicus*[33] and a similar organism[34] has not been clarified. On the other hand, it has been differentiated from toyokamycin[35], about which little published information is available, and can be distinguished from protocidine[36] and akitamycin[37] by its lack of amphoteric properties and optical rotation, respectively.

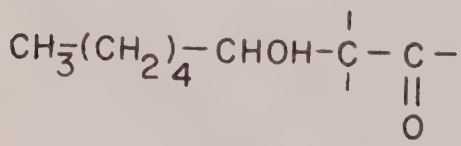
Pentaenes

Fungichromin and fungichromatin were the first antibiotics containing a pentaene chromophore to be reported[38]. Each was isolated from a different *Streptomyces* species and possessed a unique chromophoric system giving maximum light absorption at slightly different wave lengths in the visible region. The organism producing fungichromin was identified as *Strepto-*

myces cellulosae and also found to produce actinomycin. Simultaneous production of pentaenes with actinomycin and with the questiomycins, which are chemically related to the actinomycins, by other *Streptomyces* species has been noted by Japanese workers.[39,40] Recent chemical studies on fungichromin[41] have established the structure (II) of part of the fungichromin molecule

(II)

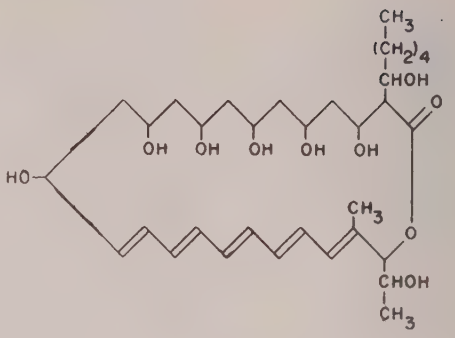
in the region of the chromophore and also shown the presence of the grouping (III)



(III)

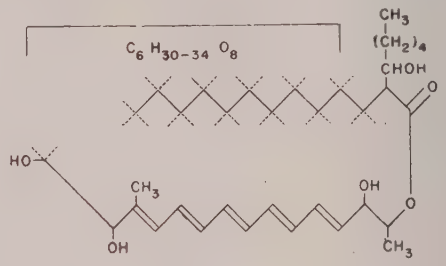
by the isolation, under conditions leading to retroaldol fission, of *n*-hexanaldehyde[42] The moiety (II) has since been identified[43] in pentamycin, an antibiotic isolated[44] from *Streptomyces pentaticus.* Differences elsewhere in the molecule are evidently responsible for the differences from fungichromin in melting point and solubility in low boiling esters. Similar differences distinguish pentamycin from filipin, a pentaene isolated from *Streptomyces filipinensis.*[45] Filipin is also readily distinguishable from fungichromin by its Rf value in certain solvents, dimorphism, and biological activity. A detailed investigation of its chemistry[46] has resulted in the tentative structure (IV) for this substance.

Lagosin,[47] a similar pentaene antibiotic, can be distinguished from filipin in melting point, absence of dimorphism, and re-



(IV)

activity with neutral periodate. Elucidation of part of the structure of lagosin (V) has



(V)

been accomplished by Whiting and coworkers[48] who have also suggested that, in view of the similar or lower $E^{1\%}_{1\ cm}$ values reported for filipin and fungichromin, these antibiotics should have molecular weights at least as high as lagosin, for which a C_{41} formula is proposed. This would require a revision of the filipin formula to approximately $C_{42}H_{70}O_{13}$, which would conform better with the results of hydrogenation experiments (see remarks by Dutcher[10]). Additional support for an upward revision of the molecular weight comes from the unusual degradation of filipin in concentrated solution to an inactive tetraene which on this basis would have an uptake of 4 moles of hydrogen on catalytic reduction over palladium on charcoal instead of the 2.9 reported.[45] Although the molecular size of pentamycin has not been reported, similarities in the analysis and $E^{1\%}_{1\ cm}$ indicate that it is probably of the same order.

TABLE II—The pentaenes

* Average of reported analyses
§Estimated from published spectra or calculated from published data
**See text for further information

Abbreviations: 2,4—DNPH —2:4 dinitrophenylhydrazine
DMF —dimethylformamide
DMAC —dimethylacetamide

Antibiotic	m.p.	Molecular composition	λmax. in mμ ($E^{1\%}_{1\,cm}$)	I.R. max. (μ) C=O str. region	(α) D	Ionizable groups	Colour reactions and functional group tests
Fungichromin	205-210°	C, 60.93; H, 8.65% **3-4 $C.CH_3$ **$C_{35}H_{60}O_{13}$	322.5§(960) 338.5§(1550) 356·5 (1430)	5.85 (6·10) 6·30	—	—	Violet → blue with conc. H_2SO_4; Doubtful Tollens
Fungichromatin	—	—	§318 (960) §333 (1550) §350 (1430)	—	—	—	Deep blue with conc. H_2SO_4.
Pentamycin	237° (d.)	C, 61.5; H, 8.4%	322 §(899) 338 (1450) MeOH 356 §(1500)	5.81	—160°(MeOH)	—	Deep purple with conc. H_2SO_4; doubtful Tollens; neg. Fehling, $FeCl_3$.
Filipin	147° & 195-205° (d.)	*C, 63.9; H, 9.0% **3-4 C.CH3 **$C_{32}H_{54}O_{10}$	322 (910) 338 (1360) MeOH 355 (1330)	5.86	—148°(MeOH)	Neutral	Blue→purple with conc. H_2SO_4; pos. Molisch; neg. ninhydrin, $FeCl_3$, 2, 4-DNPH.
Lagosin	235° (d.)	$C_{41}H_{68\text{-}70}O_{14}$	— — 356.5 (1491)	—	—	—	—
Moldcidine A	180-230°(d.)	*C, 55.8; H, 8·8 N, 1.5%	324 §(520) 80% 339 §(850) MeOH 358 §(820)	§ 5.85 § 6.35	—	Acidic	Pos. ninhydrin; neg. Fehling, Molisch, $FeCl_3$.
PA 153	—	C, 59.94; H, 8.29; N1 1 88%; 3 $C.CH_3$ $C_{37}H_{61}NO_{14}$	317 §(840) 80% 332 (1400) MeOH 349 §(1445)	§ 5.8 § 6.4	+398°(pyridine) +296° (DMF) +353°(acid DMF)	Amphoteric	Violet with conc. H_2SO_4; grey-green fluorescence in u. v.; pos. ninhydrin, Fehling, 2, 4-DNPH
Aliomycin	—	Contains C, H, O, N, S	321 330 351	—	—	—	Red-purple in conc. H_2SO_4; pos. Fehling; weak pos. Molisch, Seliwanoff.
Antibiotic from *S. effluvius*	250°(d.)	C, 58.36; H, 8.24 N, 2.04%	317 333 MeOH 350	—	+253°(HOAc)	Acidic	Violet Carr-Price; pos. Fehling; neg. ninhydrin.
Eurocidin	not <300°	*C, 57.97; H, 8·17 N, 1.65%	318 Alkaline 333 solution 350	—	—200°(0.1N HCl) +22°(0.1N NaOH)	Amphoteric	Red-purple with conc. H_2SO_4; pos. Fehling; neg. biuret, Molisch, Seliwanoff

Although none of the pentaenes described above contain nitrogen, well characterized nitrogen-containing pentaenes have been reported. Moldcidine A, isolated from the mycelium of *Streptomyces* strain 1068,[49] is such a compound and is distinguishable from antibiotic PA 153,[30] a second example of this type, by its absorption spectrum, analysis and absence of reaction with Fehling's solution. It is also different from aliomycin,[50] which is produced together with acidomycin by *Streptomyces acidomyceticus* and gives a positive Fehling test. Aliomycin is unusual in containing sulphur as well as nitrogen but since the purity of the preparation appears to be questionable, this may not represent a genuine difference from other polyene antibiotics. A nitrogen containing pentaene from *Streptomyces effluvius*[51] also differs from moldcidine A in absorption spectrum and in giving a positive Fehling test, and from both moldcidine A and PA 153 in failing to react with ninhydrin. Eurocidin, the first reported nitrogenous pentaene, was initially isolated as a crystalline compound from the mycelium of *Streptomyces alboreticuli*.[52] It has since also been obtained from *Streptomyces eurocidicus*[1] and *Streptomyces reticuli*,[53] although published evidence on the identity of all three products is inconclusive. The absorption spectrum of eurocidin indicates a closer relationship to PA 153, aliomycin and the antibiotic from *Streptomyces effluvius* than to moldcidine A from which it also differs in other respects.[49]

Hexaenes

No member of this group has yet been isolated in a convincing state of purity, although four named compounds have been described. The first antibiotic to be recognized as a hexaene was mediocidin, isolated from the mycelium of several strains of *Streptomyces mediocidicus*.[1] Flavacid,[54] which had been obtained earlier from a strain of *Streptomyces flavus*, was subsequently placed in the same group[2] but the relationship between the two has not been clarified. The existence of a hexaene chromophore in endomycin B (helixin B) was also discovered[4] only several years after initial isolation of the antibiotic[55, 56] and though it has been shown to differ paper chromatographically from flavacid, it has not been compared with mediocidin. On the other hand, cryptocidin[57] has been distinguished from both flavacid and mediocidin, though its relationship to endomycin B is unknown.

Conclusive evidence for the presence or absence of nitrogen in these substances must await their isolation in a pure state. It is also uncertain whether or not the maximum at 290, 305 and 320 mμ in the published spectra of flavacid, mediocidin and cryptocidin represent a secondary tetraene chromophore or contamination with a second tetraenic antibiotic.

Heptaenes

Ascosin, candicidin and trichomycin were among the earliest reported polyene antibiotics but as a result of difficulties encountered in their purification none has yet been described as a pure crystalline compound. Extensive studies on trichomycin isolated from *Streptomyces hachijoensis*[58] by Japanese workers have shown that the crude product contains a mixture of active factors which may be separated by countercurrent distribution[59] into two main heptaene-containing fractions, A and B, and two minor ones, C and D. Trichomycins A and B were isolated as amorphous powders unstable even in the solid state at low temperature, giving irregular analyses, and very similar in properties. Candicidin produced by a strain of *Streptomyces griseus*[60] shows paper chromatographic evidence of heterogeneity and the mobility of the complex compared with trichomycin in several solvent systems[61] suggests that some, if not all, of the components may be identical. The crude products have a virtually identical biological spectrum,[62] and are also similar in stability, solubility and spectral properties.

TABLE III—The hexaenes

§ Estimated from published spectra or calculated from published data

Abbreviations: 2,4—DNPH—2-4 dinitrophenyl-hydrazine
DMF—dimethylformamide
DMAC—dimethylacetamide

Antibiotic	m.p.	Molecular composition	$\lambda_{max.}$ in mμ $(E^{1\%}_{1\,cm})$	I.R. max. (μ) C=O str. region	$(\alpha)_D$	Ionizable groups	Colour reactions and functional group tests
Mediocidin	—	—	339-40 356-57 377-78 MeOH	—	—	—	—
Flavacid	200°(d.) 102-105° (d.) (Na salt)	C, 61·57; H, 7·77; N, 1·06; ash 2·2%	341 358 379 EtOH	—	—	Acidic	Pos. ninhydrin, $FeCl_3$; neg. Fehling.
Endomycin B	—	—	339 358 379 EtOH	—	—	Amphoteric	—
Cryptocidin	100-115° (d.)	Contains N.	341 §(350) 358 §(580) 380 §(600) 80% MeOH	§ 5.9 § 6.2	—	Acidic	Deep blue with conc. H_2SO_4; neg. ninhydrin $FeCl_3$, Fehling, Molisch.

Ascosin, which is found in both the mycelium and culture filtrates of *Streptomyces canescus*,[63] is indistinguishable in biological activity from trichomycin and candicidin[62] but paper chromatographic evidence[61] suggests that it contains a major active component different from the main fractions of either trichomycin or candicidin.

Candidin[64] and amphotericin B[22] are closely related heptaene antibiotics differing from the trichomycin-ascosin-candicidin group in their greater stability and lower solubility as well as in their biological activity and light absorption spectra. They can be distinguished from each other paper chromatographically,[61, 65] show somewhat different colour reactions in concentrated sulphuric acid, and there appears to be a difference in the degree of unsaturation since candidin takes up nine moles of hydrogen on catalytic reduction, two more than amphotericin B. The organism producing candidin has been named *Streptomyces viridoflavus*.[66] It is noteworthy that candidin and amphotericin B are the only heptaene antibiotics yet discovered which can be readily isolated from the culture and purified to a crystalline compound which is paper chromatographically homogeneous.[65] A *Streptomyces* antibiotic designated PA 150,[30] from its absorption spectrum related to the ascosin-candicidin-trichomycin series, has been shown[65] to consist of at least two components and to be chromatographically similar to antibiotic 757.[67] Chemical and physical properties have been given for a crystalline preparation of antibiotic PA 150, but no information provided to indicate whether this material was a mixture of similar heptaenes or contained only the major active component of the complex.

Streptomyces aureofaciens has been reported on several occasions[68] to produce an anti-yeast factor and a crude preparation has been named aureofacin. A complex of two heptaene antibiotics,

TABLE IV—The heptaenes

*Average of reported analyses
§Estimated from published spectra or calculated from published data
‡Solution in aqueous NaOH

Abbreviations: 2,4—DNPH 2:4 dinitrophenylhydrazine
DMF dimethylformamide
DMAC dimethylacetamide

Antibiotic	m.p.	Molecular composition	$\lambda_{max.}$ in mμ ($E^{1\%}_{1\,cm}$)	I.R. max (μ)C=O str. region	(α) D	Ionizable groups	Colour reactions and functional group tests
Ascosin	—	Low N in crude product	358 376 MeOH 399	5.87 (6·15, 6·25) 6.40	—	Amphoteric	Blue with conc. H_2SO_4; neg. ninhydrin, $FeCl_3$, Molisch, Ehrlich, carbylamine, Benedict.
Candicidin	—	Preparation with $E^{1\%}_{1\,cm}$ (380 mμ) 645 gave N 1.58-2.16%	359-62 379-81 EtOH 402-04	5.87 (6.15, 6.25) 6.40	—	Amphoteric	Blue with conc. H_2SO_4
Trichomycin A	d. > 155°	*C, 59.14; H, 8.25; N, 2.24%; low $C.CH_3$	358 — 377 ‡(767) MeOH 400 —	§ 5.72 § 5.85 §(6.15, 6.25) § 6.40	—	Amphoteric	Blue→ violet with conc. H_2SO_4; pos. Ehrlich (yellow) carbylamine, $FeCl_3$, diazocoupling; doubtful 2, 4-DNPH, Molisch; neg. ninhydrin, Fehling.
Trichomycin B	d. > 155°	*C, 59.49; H, 8.09; N, 2.16% low $C.CH_3$	358 — 377 ‡(781) MeOH 400 —				
Candidin	d. > 180°	C, 60.20; H, 8.24; N, 1.52%; $6C.CH_3$; $C_{46}H_{75}NO_{14}$	363 (980) 383 (1710) EtOH 406 (1910)	5.83 5.86 6.42	+205° (HOAc) +363° (DMF)	Amphoteric	Blue with conc. H_2SO_4; neg. 2, 4-DNPH, $FeCl_3$. Fehling,
Amphotericin B	d. > 170°	*C, 57.59; H, 8.0; N, 1.7%; $C_{46}H_{73}NO_{20}$	363 §(1000) 382 §(1650) MeOH 406 §(1860)	5.8 § 6.4	−33.5° (acid MeOH) +333 (DMF)	Amphoteric	Blue-purple with conc. H_2SO_4; pos. Molisch; neg. $FeCl_3$
PA150	—	C, 62.03; H, 7.83; N, 2.73%; 4 $C.CH_3$; $C_{54}H_{82}N_2O_{18}$	358 §(730) 377 (1033) 80% 397 §(895) MeOH	§ 5.75 § 5.85 §(6.15, 6.25) § 6.35	+294° (pyridine) +148° (DMF) −34° (acid DMF) −2590° (Na salt in MeOH)	Amphoteric	Blue with conc. H_2SO_4; pos. Fehling, 2, 4-DNPH; doubtful ninhydrin
757	—	—	361 381 EtOH 404	—	—	Acidic	—

AYF-A	—	*C, 62.55; H, 7.85; N, 2.8%	363 383 409	— (526) DMAC —	—	—	Acidic	—
AYF-B	—	*C, 62.45; H, 7.63; N, 2.8%	363 383 409	— (556) DMAC —	§ 5.92 6.35	—	Acidic	—
F—17-C	—	—	365 383 408	MeOH or EtOH	—	—	Amphoteric	Deep blue with conc. H_2SO_4.
26/1	—	—	359 380 404	EtOH	—	—	Acidic	Violet→ blue with conc. H_2SO_4.
Eurotin A	—	Contains C, H, N, O	360 380 405		—	—	—	—

designated A. Y. F.-A and -B, has been obtained from this organism[69] and separated by differential solubility in methanolic calcium chloride solution. Comparisons of the two crystalline substances with candicidin, ascosin, amphotericin B and trichomycin showed them to differ in solubility and potency. In the wavelengths at which their visible absorption maxima occur, they most resemble candidin and amphotericin B but may be distinguished by their lower extinction coefficients and by differences in elementary analyses. In common with PA 150, ascosin, candicidin and the trichomycins, A. Y. F.-B shows a sharp, intense peak in its infrared spectrum at ca. 6.25μ which is absent from the spectra of candidin and amphotericin B, and might possibly be assigned to the aromatic primary amine group known to be present in the trichomycins.

Since many of the heptaene antibiotics which have been described are actually mixtures, direct paper chromatographic comparison with previously known products is the most satisfactory method of establishing the uniqueness of a newly isolated substance. Antibiotic F-17-C, the heptaene complex isolated from the mycelium of *Streptomyces cinnamomeus* f. *azacoluta*, an organism which also produces the polypeptide antibiotic duramycin and at least one other antifungal factor, has been shown[65] in this manner to consist of two main fractions, one with an Rf value corresponding to a minor component of ascosin, the other with the same Rf as the major component of antibiotics PA 150 and 757. In the same study candimycin[70] which has been shown to be paper chromatographically distinguishable from candicidin and ascosin and similarly differentiated from the trichomycin complex,[71] and candidin and amphotericin B[61], was found to possess two components, one of which had an Rf value similar to a component common to ascosin, candicidin and trichomycin, and the other with the Rf of the minor component in

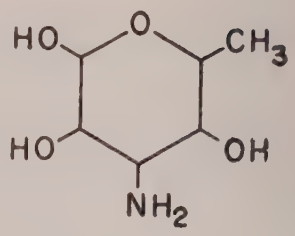
antibiotics PA 150 and 757. In the positions of their absorption maxima, antibiotic F-17-C and candimycin resemble candidin (and anti-yeast factors A and B) rather than ascosin and PA 150, so that it is possible that, in the solvent system used, components with similar Rf values may not be identical. Nevertheless, the suggestion[65] that many heptaene complexes differ quantitatively rather than qualitatively in the types of components present is probably true in many instances.

Antibiotics containing the heptaene chromophore have also been isolated from *Streptomyces viridans*[72] *Streptomyces abikoensis*[73], *Streptomyces aminophilus*[74] *Actinomyces globisporus* Kras.[75] and other unidentified actinomycetes.[3, 23, 24, 76, 77]

Chemistry

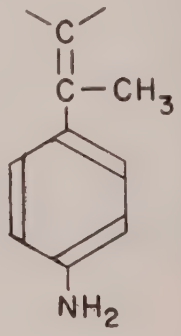
Nitrogen Function

As an alternative to classifying these antibiotics by the type of chromophore, they might be grouped according to the presence of acidic or basic functions[30] or the number of nitrogen atoms in the molecule. However, such classifications are less satisfactory since ionic properties and the molecular composition of relatively few polyenes have been established with certainty. Only a small group of pentaenes has yet been shown to be devoid of nitrogen. The majority of polyene antibiotics appear to contain one atom of nitrogen per molecule and in three examples of this type so far examined[11, 27, 78] it is present in the desoxyhexosamine substituent mycosamine[79] (VI). Vigorous acetolysis conditions are required to cleave the glycosidic bond in nystatin and amphotericin B yielding mycosamine as a mixture of its acetyl derivatives. The same sugar is liberated under milder conditions from pimaricin, and the positive iodoform reaction given by this antibiotic has been interpreted to mean that mycosamine is present in the furanose form.



(VI)

Only the heptaene PA 150 has been reported to possess two nitrogen atoms per molecule, although analytical and extinction values quoted for anti-yeast factors A and B and trichomycins A and B suggest that they may belong in this category. It is noteworthy that the trichomycins contain nitrogen in a *p*-aminophenyl substituent, possibly (VII), since it is released as *p*-aminoacetophenone on permanganate oxidation.



(VII)

Differences in the function of nitrogen containing groups in the polyenes are also suggested by reported differences in response to ninhydrin, Ehrlich, carbylamine, and other functional group tests.

Acidic Function

The majority of these antibiotics are reported to contain an acidic group. Many have been shown to be amphoteric, and it is possible that others, in which the pre-

sence of an acidic function has been deduced from the increased solubility in alkaline solution, may also contain an undetected basic group. Only in unamycin A is there firm evidence for the existence of a polyene antibiotic containing an acidic but no basic group. On the other hand, some at least of the non-nitrogenous pentaenes appear to be neutral compounds.

That a β-keto carboxyl group is responsible for the acidic properties of pimaricin has been shown by its elimination as carbon dioxide with warm, dilute sulphuric acid.[27] A carboxyl group in nystatin is indicated by the formation of a methyl ester with diazomethane[6] and also by the infrared maximum at 6.37 μ. The presence of such absorption bands in the 6.3—6.4 μ region has frequently been noted in other polyenes and is probably due in most instances to an ionized carboxyl group in the molecule. Unamycin A, which is distinguished from the tetraenes by the absence of such a band,[32] is acidic but lacks a basic function and therefore cannot exist as a "zwitter ion". It shows only a single strong absorption at 5.9 μ which may be attributed to an unionized carboxyl group.

Lactone Function

The infrared spectra of all pure polyene antibiotics which have been examined show bands in the 5.7—5.9 μ region. Dutcher and co-workers found[6,78] that this band disappeared from amphotericin B and nystatin upon saponification and, since no fragments were detected in the reaction mixture, attributed it to the presence of a lactone rather than an ester group. Structural studies on pimaricin, filipin and lagosin have shown that a macrocyclic lactone ring is present. In pimaricin it was concluded from the loss of ultraviolet absorption at 222 mμ and shift in the infrared absorption band from 5.84 to 5.77 μ on hydrogenation that the lactone carbonyl is conjugated with a double bond. In amphotericin B, on the other hand, the infrared absorption remains unchanged at 5.8 μ on hydrogenation.[78] Filipin and lagosin, since they contain a β-hydroxy lactone system, must likewise be saturated. Unamycin A may be exceptional in not possessing a macrocyclic lactone structure, but it is more probable that the very intense peak at 5.9 μ represents superimposed absorption due to both unionized carboxyl and unsaturated lactone groups.

Carbonyl other than Carboxyl or Lactone

Certain polyene antibiotics (*e.g.* PA 153 and 166) are reported to give positive reactions with 2,4-dinitrophenyl hydrazine and others (*e.g.* trichomycins A & B) give a conclusive test after catalytic reduction. The presence of reducing carbonyl groups is indicated in compounds such as PA 150, 153 and 166, eurocidin and chromin. Pimaricin has been shown[27] to contain an α, β-epoxy ketone and nystatin and rimocidin also give a characteristic test for such a group.[80] Multiple carbonyl absorption maxima at 5.7—6.0 μ in the infrared spectra of PA 150, candidin and the trichomycins suggest the presence of more than one type of carbonyl group in these compounds.

Hydroxyl Functions

Analyses of polyenes show a high percentage of oxygen and, where performed, acetylation experiments or active H values have indicated numerous hydroxyl functions to be present. The twelve, in addition to those of the mycosamine moiety, found in amphotericin B, are presumed to occur as hydroxyl substituents on the carbon chain. Likewise, nystatin appears to contain eleven,[11] filipin seven or eight[46] fungichromin ten or eleven[41] and lagosin eleven or twelve.[47] In all these compounds, only a limited number are vicinal, judging by the results of experiments with glycol-cleaving reagents. *N*-acetylpimaricin reacts immediately with one mole of periodate, yielding

formaldehyde, and slowly consumes a second.[27] Nystatin, after hydrogenation, rapidly takes up three moles of periodate with the liberation of one mole of formaldehyde.[6] Amphotericin B also consumes only two to three moles of periodate[78] In neutral or acidic solution, lagosin reacts with two moles of periodate to give an ester in which the pentaene chromophore is now conjugated with an aldehyde group. Alkaline hydrolysis then yields a neutral fragment which consumes an additional mole of oxidant to give acetaldehyde and 2-methyl-2, 4, 6, 8, 10-dodecapentaene--1,12-dial, thus establishing the nature of a considerable part of the molecule in the neighbourhood of the chromophore.[47] Fungichromin and pentamycin also yield 2-methyl-2, 4, 6, 8, 10-dodecapentaene-1, 12-dial[41, 44] when reacted with periodate after an initial treatment with hot carbonate solution, and so must possess 1,2-glycol or potential glycol groups at each end of the chromophore. Filipin, on the other hand, does not react with neutral periodate, and after alkaline treatment takes up only one equivalent of oxidant yielding acetaldehyde. It must possess only a single potential 1,2-glycol group involving the lactonic hydroxyl. The remaining hydroxyl groups are believed to be present as 1, 3-glycols.[46]

Carbon Chain Branching

Kühn-Roth oxidation of the polyene antibiotics has invariably shown relatively few terminal methyl groups to be present, and the absence of a heavy substitution within the chromophore is indicated by the position and intensity of the maxima and the fine structure evident in the ultraviolet or visible absorption spectra.[2] A fully isoprenoid carbon chain in these compounds is thus rendered unlikely but substitution by single methyl groups does occur, as in the chromophores of lagosin, fungichromin, pentamycin and filipin. Carbon chain branching elsewhere in the molecule has also been demonstrated in lagosin, fungichromin, filipin and pimaricin.

Chromophore

In addition to the chromophore-containing fragments isolated after periodate oxidation of lagosin, fungichromin and pentamycin which show the same trisubstituted pentaene to be present in each of these antibiotics[41, 43, 47], sebasic[27] and 2-methylhendecanedioic acid[46] have been obtained from the hydrogenated chromophores of pimaricin and filipin respectively. Ozonization experiments[81] on crude trichomycin, which have yielded glyoxal but no methyl glyoxal, also support the conclusion that the chromophores of the polyene antibiotics are not of the fully isoprenoid type.

Nystatin, pimaricin, PA 166 and candidin have been shown to take up, on reduction over palladium catalyst, two moles of hydrogen more than required to saturate the principal chromophore. In nystatin this was attributed to the probable presence of a secondary diene system.[11] However, subsequent work on pimaricin has indicated that this may not be the true explanation and that the disappearance of the 230 mμ peak is due to the reduction of an α, β-unsaturated lactone. The remaining mole of hydrogen is required in the reduction of an α, β-epoxy group which can be demonstrated in nystatin and rimocidin as well as pimaricin.

Antibiotics containing pentaene or heptaene chromophores can each be further subdivided into smaller groups according to the wavelength at which their maxima occur. In the pentaenes two series exist represented by fungichromin (322.5, 338.5, 356.5 mμ) and fungichromatin (318, 333, 350 mμ), while in the heptaenes there are at least two distinct groups represented by ascosin (358, 376, 399 mμ) and candidin (363, 383, 406 mμ).

Certain heptaenes (*e.g.* candicidin) show maxima at intermediate wavelengths but these have been found[61] to vary accord-

ing to the preparation examined. Such changes might be caused by variations in the composition of a complex of substances containing slightly different chromophores, or, more probably since the values shift to higher wavelengths on storage or exposure of the solution to sunlight, to *cis→trans* isomerization reactions. In aqueous dispersions of heptaene antibiotics a Tyndall effect is observed[64, 65, 82] and appears to be more pronounced, and characteristic with members of the candidin sub-group. The nature of the structural differences in the chromophores giving rise to these variations has not yet been elucidated, but it is noteworthy that in the visible spectra of heptaenes of the candidin type the relative intensities and sharpness of the absorption peaks are similar to those of tetradecaheptaene[83] whereas in the other heptaenes the absorption is less intense (cf. log Σ_{383} mμ of 5.19 for candidin vs. log Σ_{377} mμ of 5.03 for PA 150) and the maxima, as well as appearing at lower wavelengths, are of different relative intensity and have less "fine structure". Such differences might also be attributed to the presence of *cis* bonds in the chromophore.[84]

Relationship to Other Antibiotics

The Macrolides

The macrocyclic lactone structure now demonstrated for lagosin, filipin and pimaricin, and probably a common feature of the whole group of polyene antibiotics,[27] establishes a close relationship to a second series of *Streptomyces* antibiotics, the "macrolides."[85] Although these are primarily active against Gram positive bacteria, they possess many of the structural elements, such as substituent methyl, hydroxyl, epoxide, carbonyl and unsaturated groups as well as glycosidically-bound amino sugar moieties, which appear in the polyenes. Again, a large number of variations of the basic structural type, each showing differences in biological activity but probably acting by a common inhibitory mechanism, has been shown to exist.

In view of this strong family relationship, the term "macrolide" can be extended to include all of these antibiotics[27, 46, 47] the two types being distinguished, where necessary, as "antibacterial macrolides" and "antifungal" or "polyenic macrolides".

The Fradicin-Mycelin Group

One other small group of *Streptomyces* antibiotics bears a noticeable resemblance to the polyenes, in its activity, which is restricted to fungi, as well as in the poor solubility and high degree of unsaturation of the individual compounds. In addition to fradicin and mycelin, four related compounds have been reported and the series has been classified[3, 12] with the hexaenes. Although three of the visible peaks do appear at the wavelength associated with a hexaene chromophore, the spectrum is not that of a simple conjugated polyene, and the analysis of fradicin, showing the presence of methoxyl groups, a relatively low proportion of hydrogen and oxygen, and a higher proportion of nitrogen than found in the polyene antibiotics indicates that these compounds should be classed in a separate group.

Fumagillin

Unlike the polyenic macrolides which have so far only been found in various species of *Streptomyces*, fumagillin is produced by a true fungus. It is distinctly different in chemical structure[86] and should clearly be placed in a separate classification.

Flavofungin

Flavofungin has been classed as a polyene antibiotic with at least four conjugated double bonds, but appears to possess certain differences from other members of the group. Obtained from *Streptomyces flavofungini*, it has been shown to have a molecular formula $C_{30}H_{48}O_9.2H_2O$ and absorption maxima at 263 and 368 mμ. Several fragments have been identified in-

cluding two vicinal C-methyl substituents within the chromophore, an ester or lactone conjugated with the chromophore, an *n*-heptyl substituent, and a 10-carbon fragment carrying seven acetylatable hydroxyl groups.[87]

Distribution

Several surveys[3, 6, 23, 24] of the distribution of polyenes in soil actinomycetes have established that they are by far the most common group of antifungal substances produced by these organisms. The relative abundance of substances containing the different types of chromophore differs according to the soils sampled. Thus Vanek, Dolezilova and Rehacek[23] and Pledger and Lechevalier[24] found heptaenes to predominate whereas Ball, Bessel, and Mortimer[3] found a majority of tetraenes and pentaenes. The occurrence in the same organism of two or more polyenes with different numbers of conjugated double bonds is not unusual, though certainly less common than the presence of a single chromophoric type. However, it should be noted that the existence of only a single chromophore does not necessarily mean that the crude polyene is not a mixture. The production of polyenes together with other antibiotics of a different type has been noted and Vanek and co-workers, in their detailed study, found that this is actually more common than the occurrence of a polyene alone. The nature of the second antibiotic varied and was fairly evenly distributed through groups I—IV of Waksman and Lechevalier's classification.[88]

Biosynthesis

The prevalence of "macrolide" antibiotics in the actinomycetes suggests that this type of structure may have special significance for the group. However, their role is as yet obscure and little work which would have a bearing on this aspect has been reported. Dutcher and co-workers[78] have pointed out that the 40-carbon chain of amphotericin B may be related to the carotenoids and the observation[19] that mevalonic acid stimulates the production of antimycin A suggests that isoprenoid precursors are involved. However, the absence of a fully isoprenoid system does not support this type of relationship. Biosynthesis of the lactone ring from acetate with some participation by propionate and/or isoprenoid units is more likely and it has recently been shown[89] that the aglycone of one macrolide, erythromycin, is derived from propionate units. Filipin[90], fungichromin[91], and unamycin A[32] but not tennecetin (pimaricin)[28], are produced in better yield when the nutrient media contain certain oils or fatty acids. Whether this is due to provision of an abundance of activated acetate units, or whether it is merely an indirect physiological effect, has not yet been ascertained. Since cyclic and aromatic structures are now known to be formed by condensation of series of acetate and isoprenoid units, these antibiotics may in fact represent by-products of such a process, accumulated under the restricted nutritive conditions usually associated with production of high yields of secondary metabolic products by microorganisms.

REFERENCES

1. Utahara, R., *et al. J. Antibiot.* (*Japan*) **7A**, 120 (1954).
2. Oroshnik, W., *et al. Science* **121**, 147 (1955).
3. Ball, S., *et al. J. Gen. Microbiol.* **17**, 96 (1957).
4. Vining, L. C., and Taber, W. A. *Canad. J. Chem.* **35**, 1461 (1957).
5. Hosoya, S., and Hamamura, N. *J. Antibiot.* (*Japan*) **9A**, 129 (1956).
6. Dutcher, J. D., *et al. In* Therapy of Fungus Diseases. Little, Brown and Co., Toronto, 1955, p. 168.
7. Lampen, J. O., *et al. J. Bact.* **74**, 297 (1957).
8. Lampen, J. O., *et al. J. Bact.* **78**, 282 (1959).
9. Gottlieb, D., *et al. Science* **128**, 361 (1958).

10. Perritt, A. M., *et al. Biochem. and Biophys. Res. Commun.* **2**, 432 (1960).

11. Dutcher, J. D. *In* Monographs on Therapy (publ. by the Squibb Institute for Medical Research, New Brunswick, N. J., U.S.A.) **2**, 87 (1957).

12. Neelameghan, A. *Hindustan Antibiot. Bull.* **2**, 131 (1960).

13. Hazen, E. L., and Brown, R. *Science* **112**, 423 (1950).

14. Dutcher, J. D., *et al. Antibiot. Ann.* **1953-54**, 191.

15. Davisson, J. W., *et al. Antibiot. and Chemother.* **1**, 289 (1951).

16. Wakaki, S., *et al. J. Antibiot.* (*Japan*) **5**, 677 (1952).

17. Wakaki, S., *et al. J. Antibiot.* (*Japan*) **6B**, 247 (1953).

18. Raubitscheck, F., *et al. Antibiot. and Chemother.* **2**, 179 (1952).

19. Schaffner, C. P., *et al. Antibiot. Ann.* **1957-58**, 869.

20. Ehrlich, J., *et al.* Canad. Patent 514, 894 (1955).

21. Dutcher, J. D., *et al.* U.S. Patent 2,908, 612 (1959).

22. Vandeputte, J., *et al. Antibiot. Ann.* **1955-56**, 587.

23. Vanek, Z., *et al. J. Gen. Microbiol.* **18**, 649 (1958).

24. Pledger, R. A., and Lechevalier, H. *Antibiot. Ann.* **1955-56**, 249.

25. Despois, R., *et al. Giorn. Microbiol.* **2**, 76 (1956).

26. Struyk, A. P., *et al. Antibiot. Ann.* **1957-58**, 878.

27. Patrick, J. B., *et al. J. Am. Chem. Soc.* **80**, 6688, 6689 (1958).

28. Burns, J., and Holtman, D. F. *Antibiot. and Chemother.* **9**, 398 (1959).

29. Divekar, P. V. University of Tennessee, Knoxville, Tenn., U.S.A. Private communication.

30. Koe, B. K., *et al. Antibiot. Ann.* **1957-58**, 897.

31. Arcamone, F., and Perego, M. *Ann. Chim.* **49**, 345 (1959).

32. Matsuoka, M., and Umezawa, H. *J. Antibiot.* (*Japan*) **13A**, 114 (1960).

33. Umezawa, H., *et al.* Japan. Patent 5744 ('56) [per *C. A.* **52**, 9530 (1958)].

34. Taguchi, H., and Nakano, A. *Hakko Kogaku Zasshi* **35**, 145 (1957) [per *C.A.* **51**, 18101 (1957)].

35. Ishibashi, H. Reported at meeting of Japan Antibiotics Research Association, Sept. 1957 (per ref. 32).

36. Sakamoto, J. M. J. *J. Antibiot.* (*Japan*) **10A**, 128 (1957).

37. Soeda, M., and Fujita, H. *J. Antibiot.* (*Japan*) **12B**, 293, 295, 297 (1959) (per ref. 12).

38. Tytell, A. A., *et al. Antibiot. Ann.* **1954-55**, 716.

39. Maeda, K., *et. al. J. Antibiot* (*Japan*) **9A**, 125 (1956).

40. Anzai, K., *et al. J. Antibiot.* (*Japan*) **13A**, 125 (1960).

41. Cope, A. C., and Johnson, H. E. *J. Am. Chem. Soc.* **80**, 1504 (1958).

42. See footnote in ref. 46.

43. Umezawa, S., *et al. J. Antibiot.* (*Japan*) **11A**, 273 (1958).

44. Umezawa, S., *et al. J. Antibiot.* (*Japan*) **11A**, 26 (1958).

45. Whitfield, G. B., *et. al. J. Am. Chem. Soc.* **77**, 4799 (1955).

46. Berkoz, B., and Djerassi, C. *Proc. Chem. Soc.* 316 (1959).

47. Dhar, M. L., *et. al. Proc. Chem. Soc.* 148 (1958.)

48. Dhar, M. L., *et al. Proc. Chem. Soc.* 154 (1959).

49. Sakamoto, J. M. J. *J. Antibiot.* (*Japan*) **12A**, 169 (1959).

50. Igarashi, M., *et. al. J. Antibiot.* (*Japan*) **9B**, 101 (1956) [per *C. A.* **53**, 22224 (1959)].

51. Lindner, F., *et. al.*, German Patent 1,012,430 (1957) [per *C. A.* **54**, 6045 (1960)].

52. Nakazawa, J. *J. Agric. Chem. Soc. Japan* **29**, 650 (1955).

53. Thrum, I. *Naturwiss.* **46**, 87 (1959).

54. Takahashi, I. *J. Antibiot.* (*Japan*) **6A**, 117 (1953).

55. Leben, C., *et. al. Phytopath.* **41**, 23 (1951).

56. Gottlieb, D., *et al. Phytopath.* **41**, 393 (1951).

57. Sakamoto, J. M. J., *et al. J. Antibiot.* (*Japan*) **12A**, 21 (1959).

58. Hosoya, S., *et al. J. Antibiot.* (*Japan*) **5A**, 564 (1952).

59. Hattori, K., *et al. J. Antibiot.* (*Japan*) **9A**, 176 (1956).

60. Lechevalier, H., *et al. Mycologia* **45**, 155 (1953).

61. Vining, L. C., and Taber, W. A. Unpublished results.

62. Vining, L. C., *et al. Antibiot. Ann.* **1954-55**, 980.

63. Hickey, R. J., *et al. Antibiot. and Chemother.* **2**, 472 (1952).

64. Vining, L. C., and Taber, W. A. *Canad. J. Chem.* **34**, 1163 (1956).

65. Craveri, R., *et al. Antibiot. and Chemother.* **10**, 430 (1960).

66. Taber, W. A., *et al. Antibiot. and Chemother.* **4**, 455 (1954).

67. Craveri, R., and Giolitti, G. *Ann. Microbiol.* **6**, 81 (1956) [per *C. A.* **51**, 14997 (1957)].

68. See references in ref. 69.

69. Kaplan, M. A., *et al. Antibiot. and Chemother.* **8**, 491 (1958).

70. Shibata, M., *et al. J. Antibiot.* (*Japan*) **7B**, 168 (1954).

71. Hattori, K. Nagoya University, Japan. Private communication.

72. Narasimha Rao, P. L., and Uma, B. N. *Nature* **182**, 115 (1958).

73. Ueda, M., and Umezawa, H. *J. Antibiot.* (*Japan*) **9A**, 86 (1956).

74. Oswald, E. J., *et al. Antibiot. Ann*, **1955-56**, 236.

75. Tsyganov, V. A., *et al. Antibiotiki* **4**, 18 (1959),

76. Craveri, R., *et al. Antibiot. and Chemother.* **10** 306 (1960).

77. Soeda, M., and Fujita, H. *J. Antibiot.* (*Japan*) **12B**, 368 (1959). (per ref. 12).

78. Dutcher, J. D., *et al. Antibiot. Ann.* **1956-57** 866.

79. Walters, D. R., *et al. J. Am. Chem. Soc.* **79**, 5076 (1957).

80. See footnote in ref. 27.

81. Nakano, H., *et al. J. Antibiot.* (*Japan*) **9A**, 172 (1956).

82. Bartner, E., *et al. Antibiot. Ann.* **1957-58**, 53.

83. Mebane, A. D. *J. Am. Chem. Soc.* **74**, 5227 (1952).

84. Bohlmann, F., and Mannhardt, H. J. *Chem. Ber.* **89**, 1307 (1956).

85. Woodward, R. B. *In* Festchrift. Arthur Stoll. Birkhauser AG., Basel 1957, p. 524.

86. Tarbell, D. S., *et al. J. Am. Chem. Soc.* **82**, 1005 (1960).

87. Bognar, R. *Angew. Chem.* **72**, 139 (1960).

88. Waksman, S. A., and Lechevalier, H. Guide to the classification and identification of the actinomycetes and their antibiotics. The Williams and Wilkins Co. Baltimore, Md., 1953.

89. Grisebach, H., *et al. Naturwiss.* **47**, 206 (1960).

90. Brock, T. D. *Appl. Microbiol.* **4**, 131 (1956).

91. McCarthy, F. J., *et al. Antibiot. Ann.* **1954-55**, 719.

The Infectious Nature of Ribonucleic Acid of Certain Viruses and the Approach to Antiviral Chemotherapy

B. D. RAWAL
Hindustan Antibiotics Ltd., Pimpri, Near Poona

The role of nucleic acids as carriers of specific genetic characteristics of the viruses has been recognized in recent years. The bacteriophages provided this lead, but it is now known that these bacterial viruses consist of deoxyribonucleoproteins[11,22,31] whereas the plant viruses are simple ribonucleoproteins.[6,33] The animal viruses, on the other hand, contain either deoxyribonucleic acid[29] or ribonucleic acid[27] or both.[30] The present review is restricted to one of the many genetic properties of the viruses—namely, infectivity with special reference to their ribonucleic acid (RNA). The synthesis of the infective agent is, however, included in the discussion, as these two characteristics are interdependent.

Following infection with virus, two events—virus synthesis and subsequent cellular destruction—are likely to occur in the cell.[10] The experiments of Kassanis[28] have revealed that RNA acts as a precursor in virus synthesis. He compared the events in the early stages of infection of tobacco and *Nicotiana glutinosa* by tobacco mosaic virus (TMV) and its nucleic acid. It was found that the infection could be established faster with TMV-RNA as the inoculum than with the intact virus. Similarly, Sanders[42] cultivated encephalomyocarditis virus in Ehrlich ascites tumour cells and observed that in the initial stages, while no intact virus was present, infective RNA content gradually increased and was at its peak after five hours following the infection, and at that stage intact virus was detected, thus indicating that RNA had a direct role as a precursor in the synthesis of the virus.

It is also now established that nucleic acids carry the infectivity of viruses. Studies on this aspect of the RNA of plant viruses have been facilitated by the ease with which these viruses could be obtained in pure crystalline form. TMV was first crystallized[47] in 1935 and later shown to contain RNA[6]. Simple methods like precipitation of protein in cold at pH 7.0 by the addition of 30% ethanol and separating the nucleic acid in solution have been used with turnip yellow mosaic virus. Gierer and Schramm[20] applied the phenol extraction method of Morgan and Partridge for extraction of RNA from several plant viruses. This method has been extended to various animal viruses cultivated in different hosts (Table I). Fraenkel-Konrat *et al*[15] employed detergents such as sodium dodecyl sulphate (1%) or 0.002*M* EDTA-versene for denaturing virus protein and ammonium sulphate for separation of nucleic acid from plant viruses such as TMV.

In order to assign the infectivity to the RNA and to exclude the possibility that the RNA preparation may contain intact virus, several procedures have been used. Fraenkel-Konrat and co-workers[16] found that while the infectivity of the intact virus was not affected by treatment with enzyme ribonuclease, that of the RNA from TMV was lost. Further, at room temperature or at 37°C the infectivity of RNA, unlike

TABLE I.

Virus	*Method of Cultivation*	*Reference*
Mengo	Ehrlich ascites tumour cells in mice	12
West Nile	—do—	12
Encephalomyocarditis	—do—	25, 42
Encephalomyelitis	Mouse brain	19
Eastern equine encephalitis	Mouse brain; chick embryo	56
Western equine encephalitis	—do—	55
Semiliki Forest virus	Mouse brain	9
Tick-borne encephalitis	—do—	44
Murray Valley encephalitis	—do—	4
Foot-and-Mouth	Pig kidney; mice; cattle tongue	8, 36, 49, 50, 46
Polio Type I	CNS of suckling hamsters	3
Polio Type II	—do—	3
Polio	Tissue culture	43
ECHO 1	HeLa cells cultures	45
ECHO 8	—do—	23, 45
Coxsackie A-1	—do—	23
Coxsackie A-7	—fo—	45
Coxsackie B-1	—do—	23
Coxsackie B-4	—do—	45
Coxsackie B-5	—do—	45
Coxsackie	Intramuscular inoculation of suckling mice	43
Influenza	Chorioallantoic membrane	2
Influenza Type A	—do—	32
Influenza virus A	—do—	37

that of the intact virus, is labile in the presence of 0.02*M* to 0.1*M* salts.[17,20] Again, when viral-RNA is suspended in 1*M* sodium chloride the infectivity can be recovered quantitatively after 12-16 hours at 0°C unlike that of the intact virus.[9,56] The absence of intact virus or infective protein in the RNA preparation has also been shown by immunological methods. For instance, Fraenkel-Konrat *et al*[16] found that anti-TMV serum inactivated the infectious nature of the virus but not of the RNA preparation. Moreover, the infectivity of Eastern equine encephalitis-RNA was retained after treating it with equal amounts of ethanol at 37°C for 4-5 hours while that of the intact virus was completely lost.[6] Cheng[9] also noted that intact

Semiliki Forest virus was inactivated by sodium desoxycholate whereas the RNA remained unaffected. It is thus apparent that RNA contributes to the synthesis of the virus and also is itself infectious.

The success in the development of drugs effective in viral infections depends on our understanding of the mechanism of action of these compounds on the process of virus multiplication and on the metabolic activity of infected as well as uninfected host cells. Progress of investigation on the selective inhibition of virus is relative to the knowledge we gain in studies on the biosynthesis of nucleic acids and proteins. The understanding of these biosynthetic processes in turn would be enhanced by development of new inhibitors of such processes. It would, therefore, appear that attempts to screen drugs and antibiotics which inhibit viral RNA synthesis offer a more rational approach to antiviral chemotherapy than mere testing of compounds for antiviral activity. Such a line of approach is evidenced in several investigations. For example, Rafelson and co-workers[40] studying the uptake of radiophosphate by uninfected mouse brains as well as by those infected with Theiler's GD VII virus, found that the multiplication of the virus was associated with the turnover of phosphorus in the phospholipoid and total protein bound phosphorus in the brain tissue of host mice, an indication that RNA combined with host cells to synthesize virus particles. Further, by employing radiophosphate they noted that the multiplication of this virus was inhibited by 5-chlorouridine as the uptake of radiophosphate in RNA phosphate fraction was greater in the infected mouse brain than in the uninfected tissue.[41] It could, therefore, be concluded that RNA was involved in the synthesis of GD VII virus in the mouse brains and that this RNA metabolism was susceptible to the action of 5-chlorouridine.

Synthetic compounds partially inactivating influenza,[1] Eastern equine encephalitis,[26] Russian spring-summer encephalitis,[35] louping-ill[26] and vaccinia[5,53] viruses have been reported. Most of these compounds are purines and pyrimidines.

An antibiotic from *Penicillium stoloniferum* protected mice against Semiliki Forest polio Type 2 viruses in experimental infections,[38,39] while a similar or identical compound isolated from *Penicillium funiculosum* has been reported effective in Columbia SK and Semiliki forest virus infections.[43a] It is well known that broad-spectrum antibiotics such as chloramphenicol and tetracyclines and also the "macrolide" group antibiotics such as carbomycin, erythromycin, spiramycin and oleandomycin are effective in infections with the larger viruses of the psittacosis-lymphogranuloma venereum group. Oleandomycin has also moderate activity against small viruses such as influenza A and B, infectious mononucleosis and herpes zoster. A number of other compounds (Table II) isolated from cultures of actinomycetes, bacteria and fungi are also reported to have antiphagal and antiviral activity, although none of them have attained therapeutic importance.

Tamm and co-workers[52] found that alkyl derivatives of benzimidazole had low inhibitory activity against influenza viruses and that the ribosides of benzimidazoles inhibited RNA synthesis of influenza and mumps viruses.[51] The rate of inhibition could be enhanced by multiple substitution of the chlorine bond in the benzenoid ring. β-D-ribofuranosides of the chlorobenzimidazoles were more effective and the activity was specific against viral-RNA. By prior spraying of plants with azaguanine, partial inhibition of growth of cucumber virus was achieved as a result of the incorporation of 8-azaguanine in the viral RNA at the time of its synthesis in the host.[34] Todd,[54] however, believes that this inhibitory activity of the compound may be due to the fact that incorporation of such inhibitors may produce abnormal nucleic acids resulting in the inactivation of the virus. Indeed, he recommends trials with

TABLE II.*

Virus inhibited	*Antibiotics screened*
	ANTIBIOTICS FROM BACTERIA
Influenza PR-8 and New Castle disease virus in embryonated eggs	Subtilin
Marked inhibition of infectious bronchitis virus of fowls and slight suppression of influenza and New Castle disease virus, in chick embryo	Viscosin
Influenza A and B, New Castle disease and mouse pneumonitis	Xerosin (APM factor)
	ANTIBIOTICS FROM ACTINOMYCETES
Western and Eastern encephalitis virus (1:8,000,000 dilution)	Abikoviromycin
Japanese B encephalitis in mice (1:16,000 crude ppn.)	Achromoviromycin
Y-SK poliomyelitis in tissue culture. Moderately virucidal to eastern equine encephalomyelitis and slightly so to PR-8 influenza. Several bacteriophages	Aklavin
Influenza virus PR-8 in chick embryo	Antibiotic 1-8ld-ls
Influenza A partially *in vitro*, highly effective in chick embryo	Antibiotic 719
Encephalomyelitis virus	Antibiotics 1121 and 452-7
Inactivates TMV, influenza and smallpox viruses	Antivirubin
Bacteriophages. PR-8 and FM-1 influenza virus in chick embryo	Cardicin
Japanese B encephalitis *in vitro* and *in vivo*	Cephalomycin
Virus. Bacteriophages	Cerulomycin
Bacteriophages	Chrysomycin
Influenza A virus	Cinerubine A, B
Influenza B in mice and in chick embryo	Ehrlichin
Influenza virus at 20 g./ml.	Flavucidin
Virus of silkworm jaundice	Grassenriomycin
Influenza A in mice	Heliomycin
Meningopneumonitis virus in milk	Hygromycin
Influenza virus in tissue culture at 10 μg/ml.	Hygroscopins, A, B
Virus in silkworm jaundice	Luridin
Influenza virus in mice (moderately active)	Luteomycin
Influenza A partially in vitro, highly effective in chick embryo	Antibiotic M II
Influenza virus *in vitro*	Myxoviromycin
"WR" strain vacinia virus in mice	Netropsin

Virus inhibited	*Antibiotics screened*
New Castle virus in chick embryo (0.01 mg./ml. for A and 0.75 mg./ml. for B). Influenza virus in embryonated eggs	Niromycins, A. B.
Systemic infection with Southern bean mosaic virus in Pinto bean plant and TMV in tobacco	Noformicin
Bacteriophages	Nybomycin
Bacteriophages	Phagocidin
Bacteriophages. Virus of vaccinia. Herpes simplex and rabies *in vitro*. Slight activity against Theiler and Lansing strains of poliomyelitis. Y-SK poliomyelitis in tissue culture (5 mg./ml.). Influenza A virus in chick embryo	Phagolessin A-58
Bacteriophages	Phagomycin
Bacteriophages	Phagostatin
Herpes simplex inactivated at 2 mg./ml.	Puromycin
Viruses	Violarin
Bacteriophages	Antibiotic X-465
Virus of silkworm jaundice	Virusin 1609
Rabies virus in mice	Virocidin
	ANTIBIOTICS FROM FUNGI
Phages of *M. pyogenes* and *V. comma*. Y-SK poliomyelitis partial inhibition in tissue culture at 4-8 mg./ml.	Drosophillin A
Influenza PR-8 virus (2 mg./ml.), inactive in chick embryo	Drosophilin B (Pleuromutilin)
Bacteriophages. Eastern and Western encephalitis and influenza PR-8 viruses in tissue culture at 25 mg./ml. Lansing strain of poliomyclitis virus at 0.5 mg./ml.	Rumagillin
Columbia SK encephalomyelitis. Semiliki Forest virus Western equine encephalomyelitis in mice. Mahoney strain of poliomyelitis Type 1 in monkeys	Helenine (Probably same as Antibiotic 8450)
TMV *in vitro* and to a lesser extent in isolated leaves of *Nicotiana glutinosa*	Antibiotic from *Mortierella* spp.

* A detailed list of antiviral antibiotics will be published in a future issue of *Hindustan Antibiotics Bulletin*.

"small synthetic oligonucleotides containing in them abnormal nucleotides." These lines of approach are in harmony with the "rational approach" of Staehlin[48] who saw a hope of antiviral chemotherapy using antagonists for either ribose or uracil. Although several trials, based on different theories, are progressing, considerably more knowledge of viruses and their chemical nature is needed before successful antiviral chemotherapy can be achieved.

REFERENCES

1. Ackermann, W. W. *Proc. Soc. Exp. Biol. Med.* **80**, 362 (1952).
2. Ada, G. L., and Perry, B. T. *Aust. J. Exp. Biol. Med. Sci.* **32**, 453 (1954).
3. Alexander, H. E., *et al. Virology* **5**, 172 (1958).
4. Anderson, S. G., and Ada, G. L. *Aust. J. Exp. Biol.* **37**, 353 (1959).

5. Bauer, D. J. *Brit. J. Expt. Path.* **36**, 105 (1955).
6. Bawden, F. C., and Pirie, N. W. *Proc. Roy Soc.* **123B**, 274 (1938).
7. Brown, F., and Stewart, D. L. *Virology* **7**, 408 (1959).
8. Brown, F., *et al. Nature* **182**, 535 (1958).
9. Cheng, P. O. *Nature* **181**, 1800 (1958).
10. Cheo, P. C., *et al. Proc. Natl. Acad. Sci.*, **45** 305 (1958).
11. Cohen, S. S. *Bact. Rev.* **15**, 131 (1951).
12. Colter, J. S. *Prog. Med. Virol.* **1**, 1 (1958).
13. Cooper, P. D. *Virology* **1**, 397 (1955).
14. DeSomer, P., *et al. Nature* **184**, 652 (1959).
15. Fraenkel-Konrat, H. *Fed. Proc.* **16**, 810 (1957).
16. Fraenkel-Konrat, H., *et al. Biochim. Biophys. Acta* **25**, 87 (1957).
17. Fraenkel-Konrat, H., *et al. In* McElroy, W. D., and Glass, D. B., *eds.* The Chemical Basis Of Heredity. Baltimore, Md., John Hopkins Press, 1957, p. 501.
18. Franklin, R. M., and Wecker, E. *Nature* **184**, 343, 1959.
19. Franklin, R. M., *et al. Virology* **7**, 220 (1959).
20. Gierer, A., and Schramm, J. *Nature* **177**, 702, (1956).
21. Ginsberg, H. S. *Bact. Rev.* **24**, 14 (1960).
22. Hershey, A. D., *et al. J. Gen. Physiol.* **36**, 777 (1953).
23. Holland, J. J., *et al. Proc. Soc. Exp. Biol. Med.* **100**, 843 (1959).
24. Holland, J. J., *et al. J. Exptl. Med.* **110**, 65 (1959).
25. Huppert, J., and Sanders, F. K. *Nature* **182**, 515 (1958).
26. Hurst, E. W., *et al. Brit. J. Pharmacol.* **7**, 455 (1952).
27. Hyden, H. Cold Spring Harbour Symp. Quant. Biol. **12**, 104 (1947).
28. Kassanis, B. *J. Gen. Microbiol.* **20**, 704 (1959).
29. Knight, C. A. Cold Spring Harbour Symp. Quant. Biol. **12**, 315 (1947).
30. Knight, C. A. *J. Exp. Med.* **85**, 99 (1947).
31. Kozloff, L. M. *Exp. Cell. Res. Suppl.* **2**, 367 (1952).
32. Maassab, H. F. *Proc. Natl. Acad. Sci.* **45**, 877 (1959).
33. Markham, R., *et al. Nature* **162**, 88 (1948).
34. Mathews, R. R. F. *In* Ciba Foundation Symposium on the Chemistry and Biology of Purines. London, J. and A. Churchill, 1957, p. 270.
35. Moore, A. E., and Friend, C. *Proc. Soc. Exp. Biol. Med.* **78**, 153 (1951).
36. Mussgay, M., and Strohmaier, K. *Zentr. Bakteriol. Parasit., Orig.* **173**, 163 (1958).
37. Portocala, R., *et al. Compt. rend.* **249**, 201, 848 (1959).
38. Powell, H. M., and Culbertson, C. G. *Proc. Soc. Exp. Biol.* **83**, 161 (1953).
39. Powell, H. M., *et al. Antibiot. and Chemother.* **2**, 432 (1952).
40. Rafelson, M. E., Jr., *et al. Arch. Biochem.* **29**, 69 (1950).
41. Rafelson, M. E., Jr., *et al. Proc. Soc. Exp. Biol. Med.* **76**, 689 (1951).
42. Sanders, F. K. *Nature* **185**, 802 (1960).
43. Schaffer, F. L., and Mattern, C. F. T. *Fed. Proc.* **18**, 317 (1959).
43a. Shope, R. E. *J. Exp. Med.* **97**, 601, 639 (1953).
44. Sokol, F., *et al. Nature* **184**, (*Suppl.* 20) 1581 (1959).
45. Sprunt, K., *et al. Proc. Soc. Exp. Biol. Med* **101**, 604 (1959).
46. Spuhler, V. *Experientia* **15**, 155 (1959).
47. Stanley, W. M. *Science* **81**, 644 (1935).
48. Staehlin, M. *Prog. Med. Virol.* **2**, 1 (1959).
49. Strohmaier, K., and Mussgay, M. *Z. Naturforsch.* **14b**, 171 (1959).
50. Strohmaier, K., and Mussgay, M. *Science* **130**, 217 (1959).
51. Tamm, I. *Science* **120**, 847 (1954).
52. Tamm, I, *et al. Yale J. Biol. Med.* **24**, 559 (1952).
53. Thompson, R. L., *et al. J. Immunol.* **67**, 483 (1951).
54. Todd, A. *Brit. Med. J.* **2**, 517 (1960).
55. Wecker, E. *Virology* **7**, 241 (1959).
56. Wecker, E., and Schafer, W. *Z. Naturforsh.* **12b**, 415 (1957).

Some *Streptomyces* species Producing Oxytetracycline

M. J. THIRUMALACHAR & V. V. BHATT ‡

Research Laboratories, Hindustan Antibiotics Ltd., Pimpri Near Poona

Considerable attention has been focussed in recent years on the isolation and study of microorganisms producing valuable metabolic products, particularly antibiotics. These are of interest both from the commercial and academic points of view. Oxytetracycline, an important broad-spectrum antibiotic, is known to be produced by several species of *Streptomyces*. Sobin *et al.*[5] first described the production of oxytetracycline from *Streptomyces rimosus*, and since then, several other species have been described which produce this antibiotic in submerged cultures. These include *S. alboflavus, S. armillatus, S. gilvus, S. griseoflavus, S. griseolus, S. utilis, S. vendargensis* and *S. vorosoviensis*. Many of these species are poorly described, the description being combined with the patent specifications for the manufacturing process. Comparative mycological study of some of these species is almost impossible since the type cultures are not available and only published descriptions have to be taken into account.

In the course of our studies on soil actinomycetes, several *Streptomyces* species were isolated, which on further examination were found to be *S. rimosus*, often with variations. Among several other species studied, two produced oxytetracycline in submerged culture but differed from *S. rimosus* and other species listed above, which also produce this antibiotic. One of these was designated *S. utilis* Thirum.,* and the other, culture No. 27-A, was also found to be an undescribed species of *Streptomyces*. A description of the latter is presented in this paper.

The chief characteristics of *S. utilis* have already been given* and only a brief mention is made here for purposes of comparison. The second species studied (culture No. 27-A) produces white aerial mycelium bearing numerous sporophores with monoverticillate spirals. *S. griseolus* and *S. platensis* produce grey coloured aerial mycelium and are therefore distinct. *S. alboflavus* and *S. griseoflavus* also produce white powdery aerial mycelium, but the sporophores are straight and do not form spirals. *S. vendargensis*[1] and *S. armillatus* also form white aerial mycelium, and the former produces the antibiotic vengicide, in addition to oxytetracycline. *S. armillatus* differs from the one under study in its inability to reduce nitrates and failure to hydrolyse starch and in other characters.[2] A comparative account of the growth characters on different media is given in Table I.

Utilization of carbon compounds were studied using the method described by Pridham and Gottlieb.[3] The results of testing are indicated in Table II.

‡ Present address: Lederle Laboratories India (P.) Ltd., P. O. Atul, via Bulsar.

* Indian patent 1960.

TABLE I

CULTURAL CHARACTERS OF *STREPTOMYCES* 27-A, *S. UTILIS* AND *S. RIMOSUS*

Media	*27A*	*S. utilis*	*S. rimosus*
1) Synthetic agar (Czapeks)	Growth moderate, with pale yellow submerged mycelium and white aerial mycelium along border. Sporophores with spirals in monoverticillate branches. No soluble pigment.	Profuse growth, colonies with pinkish vegetative mycelium and whitish powdery aerial mycelium with few open spirals. No soluble pigment. No cracked surface on colonies.	No growth
2) Glucose asparagine agar	Growth profuse, with abundant cottony-white aerial mycelium. No soluble pigment diffusing.	Growth moderate, and flat, with only submerged mycelium and no aerial hyphae	Growth moderate, with submerged mycelium white and "pallid Quaker Drab". Very faint yellow pigment present, reverse ochre-yellow, spirals numerous.
3) Emerson's medium	Profuse growth, mycelium mostly submerged, with n soluble pigment. Colony reverse pale cream coloured. Aerial mycelium white, with numerous spirals	Growth good, only submerged mycelium, pink coloured, with no soluble pigment	Growth moderate to good, colony surface cracked by fissures, colonies *en mass* honey-yellow, pale-yellowish pigment diffusing; aerial mycelium white to "pallid Quaker Drab"
4) Calcium malate agar	Profuse growth, submerged mycelium, pale white, without soluble pigment. Aerial mycelium powdery white with numerous spirals	Growth moderate, translucent, pink, no soluble pigment diffusing and no aerial mycelium	Growth poor, flat, colony colour yellow without soluble pigment or aerial mycelium
5) Starch agar	Growth good, mycelium submerged transparent, with fluffy white aerial mycelium in patches	Growth moderate, mycelium submerged and pale pinkish	Growth poor, thin, very little aerial mycelium, no soluble pigment
6) Potato plug	Growth profuse, white powdery aerial mycelium no soluble pigment diffusing, potato turning slightly dark-brown	Growth good, translucent, cream coloured no aerial mycelium or spores	Growth moderate, wrinkled, aerial mycelium, abundant, pale yellowish-brown pigment diffusing
7) Starch hydrolysis	Strong	Strong	Moderate
8) Nitrate reduction	Positive	Positive	Positive
9) Gelatin liquefaction	Strongly liquefied, submerged growth, pinkish with pale brown pigment diffusing	Not liquefied, growth moderate with white aerial mycelium and pinkish submerged growth, pale-brown pigment diffusing	Moderately liquefied, growth whitish, without soluble pigment diffusing
10) Litmus milk	Fair growth and slight coagulation	Poor growth and no change in litmus milk	Growth good, no hydrolysis or peptonisation

TABLE II.

UTILIZATION OF CARBON COMPOUNDS BY *STREPTOMYCES* 27-A, *S. UTILIS* AND *S. RIMOSUS*

Carbon source	*27-A*	*S. utilis*	*S. rimosus*
Arabinose	+	++	+++
Aesculin	++	+	—
Dulcitol	—	—	—
Galactose	+++	+++	+++
Glycerol	+++	+++	+++
Glucose	+++	+++	+++
Lactose	+	+	+++
Levulose	+++	+++	++
Maltose	+++	+++	+++
Mannose	+++	+++	+++
Mannitol	+++	+++	+++
Raffinose	+++	+++	+
Rhamnose	++	+	—
Salicin	+++	+	—
Soluble starch	+++	+++	+++
Sorbitol	+++	+++	+++
Sucrose	+	+	—

— = No growth; + = Poor growth; ++ = Fair or moderate growth: +++ = Good growth.

From the cultural characters enumerated above, *Streptomyces* species designated 27-A, is distinct from *S. rimosus* and other species producing oxytetracycline. From the point of view of morphology of the sporophores, which is considered an important diagnostic character by Pridham *et al*,[4] the species studied would come under the section "Monoverticillus-spira." Among the white series in this section, only one species is known, *S. circulatus* which is distinctly different. The name *Streptomyces albofaciens* Thirum. and Bhatt is proposed for accommodation of the new species.

REFERENCES

1. N. V. Koninklijke Nederlandsche Gist-en Spiritusfabriek. Brit. Patent 764, 198 (Dec. 9, 1956).

2. Mancy-courtillet, D., and Pinnert-Sindico, S. *Ann. Inst. Pasteur* **87**, 580 (1954).

3. Pridham, T. G., and Gottlieb, D. *J. Bact.* **56**, 107 (1948).

4. Pridham, T. G., *et al. Appl. Microbiol.* **6**, 52 (1958).

5. Sobin, B. A., *et al.* U. S. Patent 2,516,080 (1950).

Chlortetracycline Biosynthesis in Synthetic Medium

W. V. LAVATE

Research Laboratories, Hindustan Antibiotics Ltd., Pimpri, Near Poona

High yields of chlortetracycline can be obtained with *Streptomyces aureofaciens* grown on fermentation media containing crude and complex ingredients, but the process does not provide precise information either on the metabolism of the strain used or on the mode of biosynthesis of the antibiotic. Chemically defined synthetic media serve as the ideal experimental systems for studies on biosynthesis and for systematic follow-up of the course of a fermentation.

Isotopic tracer studies[4, 5, 6] have shown that (1-C^{14}) acetate and (2-C^{14}) acetate are good precursors of labelled tetracyclines and reduction of 5 $\propto$ (11 $\propto$)-dehydrotetracycline may be the final step in the biosynthesis of the tetracycline molecule. However, present knowledge about the mechanism of biosynthesis of chlortetracycline by *S. aureofaciens* and the role and chemical nature of the moieties involved therein are rather empirical and the few literature reports[1, 2, 3] are concerned only with certain aspects of the problem.

In the present studies, growth of and formation of chlortetracycline by, the strain *Streptomyces aureofaciens* AF-19 were followed in various synthetic media. The synthetic media consisted of a basal medium containing starch, $(NH_4)_2SO_4$, NaCl, $CaCO_3$, $MgSO_4$, $ZnSO_4$, $FeSO_4$, $MnSO_4$ and was supplemented with other additions like inorganic phosphate (as K_2HPO_4) and various amino acids. The experiments were carried out at 28° C. in 500 ml. Erlenmeyer flasks on a rotary shaker (250 r.p.m. and 2″ throw) The following amino acids were examined for their effect on growth and chlortetracycline formation when added to the basal medium containing 0.9 mg. per cent of inorganic phosphorus : Glutamic acid, glycine, cystine, asparagine, methionine, alanine, tryptophan, tyrosine, threonine, serine, aspartic acid, phenylalanine, histidine, valine, norvaline, leucine, isoleucine, norleucine, ornithine, lysine, arginine and glutamine. The amino acids were used as L acids or DL acids depending upon availability. L acids were added at 0.1 per cent level and the DL acids at double the concentration. These amino acids affected the growth of the organism and the chlortetracycline formation to different extent. Tyrosine was observed to exhibit a marked stimulatory effect on synthesis of chlortetracycline without significant increase in mycelium formation. Phenylalanine was also found to be effective but not to the same extent as tyrosine.

Inorganic phosphorus content of medium has been reported to have a marked influence on the biosynthesis of chlortetracycline.[6, 7] The phosphate sensitivity of *S. aureofaciens* has also been shown to be strain-specific. This aspect was studied with the strain *S. aureofaciens* AF-19 in experiments in which different amounts of inorganic phosphorus were added to the basal and amino acid supplemented media. A level of 3.8 mg. per cent of inorganic phosphorus was found to be optimum for this strain. These observations were also confirmed by additions of graded concentrations of clarified corn-steep liquor solution whose inorganic (free)

phosphorus content was previously estimated by the method of Taussky and Shorr.[12]

By studying several combinations of inorganic phosphorus levels and tyrosine concentrations, it was observed that as much as 650 μg./ml. of chlortetracycline was produced in 72 hr. with 3.8 mg. per cent of inorganic phosphorus and 0.1 per cent of l-tyrosine in the medium. Addition of benzylthiocyanate to this medium increased the titre to 800 μg./ml. Similar results were also obtained with phenylalanine but the titres were comparatively lower.

The observed stimulatory effect of tyrosine and phenylalanine on chlortetracycline formation shows that these amino acids have some intimate role in the biosynthesis of the antibiotic. The structural similarity between tyrosine and phenylalanine further suggests that they may have a common function and that the greater effectiveness of the former may be due to its better availability for or efficiency of, utilization than the latter. It is also probable that the phenyl portion of their molecules has a more important role than the propionic acid residue since propionic acid alone fails to exert any effect.

Further work to elucidate the significance of the above observations is underway.

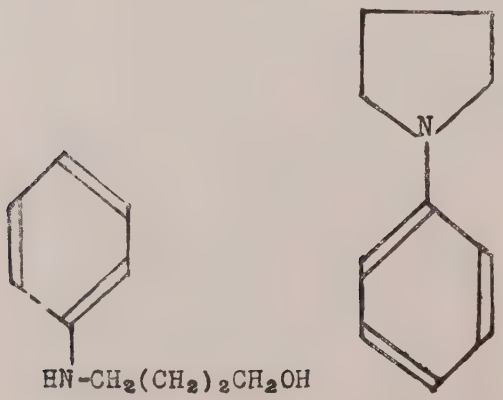
REFERENCES

1. McCormick, J. R. D., *et al. J. Bact.* **77**, 475 (1959).
2. Ling Wang, E. *J. Antibiot.* (*Japan*) **12A**, 31 (1959).
3. Petty, M. A., *et al.* VI Congresso Internazionale di Microbiologia, Roma. Riassunto della communicazioni **1**, 156 (1953).
4. Snell, J. F., *et al.* Proc. International Conf. on the Peaceful Uses of Atomic Energy, Geneva. Aug. 8-20, 1955. New York, United Nations, **12**, 431 (1956).
5. Miller, P. A., *et al. Science* **123**, 1030 (1956).
6. McCormick, J. R. D., *et al. J. Am. Chem. Soc.* **80**, 6460 (1956).
7. Di Marco, A. *G. Microbiol.* **2**, 285 (1956).
8. Prokof'eva-Belgovskaya, A., and Popova, L. *J. Gen. Microbiol.* **20**, 462 (1959).
9. Guberniev, M. A., *et al. Antibiotiki* **4**, 37 (1959).
10. Makarevich, V. G., and Laznikova, T. N. *Antibiotiki* **4**, 43 (1959).
11. Doskocil, J., *et al. J. Biochem. Microbiol. Technol. Engr.* **1**, 261 (1959).
12. Taussky, H. M., and Shorr, E. *J. Biol. Chem.* **202**, 675 (1953).

Hydroxyalkylation of Primary Aromatic Amines with Diols in the Presence of Raney Nickel

N. V. BRINGI & P. V. DESHMUKH
Research Laboratories, Hindustan Antibiotics Ltd., Pimpri, Near Poona.

Raney nickel induced *N*-alkylation of primary amines with primary and secondary alcohols has been studied by several workers.[1-5] In connection with the preparation of amine salts of penicillin,[6] we investigated the preparation of various substituted amines by reductive alkylation with Raney nickel and alcohols. In the course of this work aniline was treated with 1:4-butanediol and 1:5-pentanediol with Raney nickel in refluxing benzene solution. The reaction of 1:4-butanediol with aniline gave readily, in one step, 4-anilino-1-butanol (I) in 15 per cent yield. In a similar way 5-anilino-1-pentanol was obtained in 14 per cent yield. The structure (I) was established from the fact that in the attempt to prepare the chloride with thionyl chloride in chloroform solution, the product cyclised during work-up to yield *N*-phenylpyrollidine (II), characterised by deep red colour with nitric acid, and through the preparation of picrate.



I II

4-Anilino-1-butanol has been prepared by refluxing aniline with 4-chlorobutanol in water containing calcium carbonate.[7] Kon and Roberts[8] refluxed aniline and 4-chlorobutyl acetate in water containing calcium carbonate and subsequently hydrolysed the acetate, obtained in 25 per cent yield, to give (I). They also prepared 5-anilino-1-pentanol from aniline and pentamethylene chlorohydrin. Holmen and Carrol[9] obtained (I) in 24 per cent yield by refluxing tetramethylene chlorohydrin and aniline in benzene for 20 hr., while a 42 per cent yield of the crude product was obtained by lithium aluminium hydride reduction of succinanilic acid. In a patent, Winberg[10] described the preparation of (I) in 62 per cent yield by high pressure reduction of 2-phenyl-3, 6-dihydro-1, 2-(2H) oxazine.

The method now described for the alkylation of amines with diols in the presence of Raney nickel, represents a comparatively facile, one-step synthesis of the hydroxyalkylanilines. Two instances have been reported in literature wherein diols have been condensed with diamines in the presence of Raney nickel, to yield cyclic compounds. Kao, Tilak and Venkataraman[1] obtained 1, 2, 3, 4-tetrahydroquinoxaline in 20 per cent yield by heating *o*-phenylenediamine and ethylene glycol in the presence of Raney nickel. A somewhat similar reaction was carried out by Rice,

Kohn and Daasch[5] by heating 1:6-diaminohexane and 1:4-butanediol to obtain N-(4-hydroxybutyl) hexahydroazepine in 15 per cent yield.

EXPERIMENTAL†

4-Anilino-l-butanol (I)

A mixture of aniline (24.2 g.) and 1:4-butanediol (18.6) in dry benzene (120 ml.) was refluxed with Raney nickel* (40 g.) for 25 hr. after addition of one drop of 1 *N* aq. sodium hydroxide. The mixture was filtered, made strongly acidic and extracted with ether to remove neutral material. The aq. layer was made strongly alkaline, extracted with ether and the product obtained after removal of ether, was fractionally distilled. Following a forerun** (11.4 g.), 4-anilino-1-butanol distilled at 144°/1 mm. (6.4 g.) ; yield 15 per cent.

N-phenylpyrrollidine (II)

A soln. of 4-anilino-1-butanol (3.35 g.) in anhydrous chloroform (10 ml.) was added drop by drop to thionyl chloride (5.5 ml.) kept at 0-10°. The soln. turned brown during addition. The reaction mixture was stirred for 2 hr. at room temp. Absol. alcohol (10 ml.) was gradually added and then refluxed for 2 hr. Dilution with petrol ether (b. p. 40-60°) and cooling gave a partly crystalline and partly oily product. Petrol ether was decanted, the residue dissolved in water and made strongly alkaline. The product obtained by ether extraction of the aqueous alkaline solution was fractionally distilled. A colourless liquid distilled over at 94°/1 mm. (fraction 1) and another fraction was collected at 150°/1 mm. (fraction 2).

Fraction 1 gradually turned brown. The substance gave an intense red colour with concentrated nitric acid, which is characteristic of *N*-phenylpyrrollidine. The picrate prepared in ethanol solution, crystalised in yellow needles from ethanol, m.p. 114°.

The boiling point and analysis indicated fraction 2 to be the unreacted alcohol.

5-Anilino-l-pentanol

A mixture of aniline (12.1 g.) and 1:5-pentanediol (10.4 g.) in dry benzene (60 ml.) was refluxed with Raney nickel (20 g.) for 30 hr. after addition of one drop of 1 *N* aq. sodium hydroxide. The mixture was filtered, acidified, and extracted with ether. The aq. layer was made alkaline, extracted with ether and the residue fractionally distilled. After a forerun (4.2 g.) a colourless liquid distilled at 150-60°/0.6 mm. (2.5 g.); yield 14 per cent. Redistillation gave a liquid boiling at 162°/1 mm.

p-Toluenesulfonyl derivatives of 5-anilino-l-pentanol

5-Anilino-l-pentanol (208 mg.) in pyridine (1.5 ml.) was treated with *p*-toluenesulfonylchloride (316 mg.) and kept overnight at 5°. The wine red solution was poured on to crushed ice and the precipitated solid filtered. (yield 210 mg., ; m.p. 114-15°). The analytical sample obtained in colourless needles by crystallisation from methanol had m.p. 121°. Anal.: Calcd. for $C_{18}H_{23}NO_3S$: C, 64 88; H. 6.90 ; N, 4 20. Found : C, 64,60 ; H, 6.85 ; N, 4.25.

ACKNOWLEDGEMENT

The authors wish to thank Dr. M. J. Thirumalachar and Dr. D. S. Bhate for their encouragement and Mr. A. V. Patankar for the microanalysis.

† Satisfactory analyses have been obtained for all the compounds.

* Raney nickel was prepared in the active form from Nickel-Aluminium Alloy (B.D.H.) according to the method described in A. I. Vogel's A text book of practical organic chemistry. 3d ed. Longmans, Green and Co., 1956, p. 871.

** Analysis of the crude mixture by Hinsberg method indicated the presence of aniline (50 per cent) and secondary and tertiary amines.

REFERENCES

1. Kao, G. N., *et al. J. Sci. Ind. Res.* (*India*) **14B** 624 (1955).
2. Rice, R. G., and Kohn, E. J. *J. Am. Chem. Soc.* **77**, 4052 (1955).
3. Ainsworth, C. *J. Am. Chem. Soc.* **78**, 1635 (1956).
4. Horyna, J., and Cerny, D. *Chem. Listy* **50**, 381 (1956). *Coll. Czech. Chem. Communs.* **21**, 906 (1956).
5. Rice, R. G., *et al. J. Org. Chem.* **23**, 1352 (1958).
6. For a recent classified compilation of known amine salts of penicillin, *see* Vaidya, S. S, *Hindustan Antibiot. Bull.* **2**, 89 (1960).
7. Everett, J. L., and Ross, W. C. J. *J. Chem. Soc.* 1972 (1949).
8. Kon., G. A. R., and Roberts, J. J. *J. Chem. Soc.* 978 (1950).
9. Holmen, R. E., and Carroll, D. D. *J. Am. Chem. Soc.* **73**, 1859 (1951).
10. Winberg, H. E. U. S. Patent 2,628,978, (Feb. 17, 1953). *C. A.* **48**, 1429f (1954).

Countercurrent Distribution Studies on Phenoxymethyl Penicillin (Penicillin V)

S. B. THADANI, G. SEN & D. GHOSH
Research Laboratories, Hindustan Antibiotics Ltd., Pimpri, Near Poona

In connection with our investigation on biosynthetic acid-stable penicillins[1] countercurrent distribution technique is routinely employed for testing the homogeneity and purity of the compounds. Craig's[2] pioneer observations on distribution behaviour of penicillins were followed by studies of Behrens *et al.*,[3] Bartels and Dolliver[4] and Eisenlohr,[5] who used different solvent phases for the extraction of penicillins. More recently Sheehan and Henery-Logan[6] purified synthetic penicillin V by a 2-funnel countercurrent distribution followed by successive extraction operations in separatory funnels. To the best of our knowledge a detailed study on the distribution of penicillin V in different solvent mixtures has not been reported so far. It is well known that distribution for a small number of transfers between one pair of solvents may lead to erroneous results. Moreover, penicillin V, obtained from fermentation processes, may contain a small amount of penicillin G and in some cases other impurities as well.[7] This prompted us to undertake study of the purity of commercial penicillin V by the technique of countercurrent distribution in different solvent systems.

The solvent phases employed were : (1) Butyl acetate and citrate buffer (pH 5.7) ; (2) methyl *iso*butyl ketone-chloroform (8:2) and citrate buffer (pH. 5.7) ; and (3) methyl *iso*butyl ketone-phosphate buffer (pH 6.0) and ammonium sulphate.[6]

Penicillin was estimated by the modified iodometric method[8] as well as by spectrophotometric method measuring the absorption at 260 mμ for penicillin G and at 275 mμ for penicillin V after conversion with 1*N* NaOH.[9] Microbiological estimations were made by the serial dilution method in the usual way.

The solvents employed were distilled and methyl *iso*butyl ketone was purified by treatment with $AgNO_3$[10] followed by distillation.

Distribution operations were carried out in a 50 tube semi-automatic Craig's apparatus (Quickfit), the volume of each phase used in individual tubes being 25 ml. After each run, the pH of the lower phase was adjusted to 2.0 by the addition of 15 ml. of 20 % phosphoric acid to individual tubes. After agitation for 5 min., the phases were allowed to attain equilibrium. The organic phase from each tube was withdrawn followed by re-extraction to aqueous phase (0.1M phosphate buffer, pH 7.0) and suitable aliquots were then estimated according to the methods mentioned above.

When distributed between *n*-butyl acetate and citrate buffer, pH 5.7, potassium penicillin V showed a minor hump at tube No. 1 followed by a peak due to penicillin V at tube No. 14 [K = 1.4 ; n (transfer numbers) = 24]. It was found that there was good agreement between the theoretical and the experimental curves.

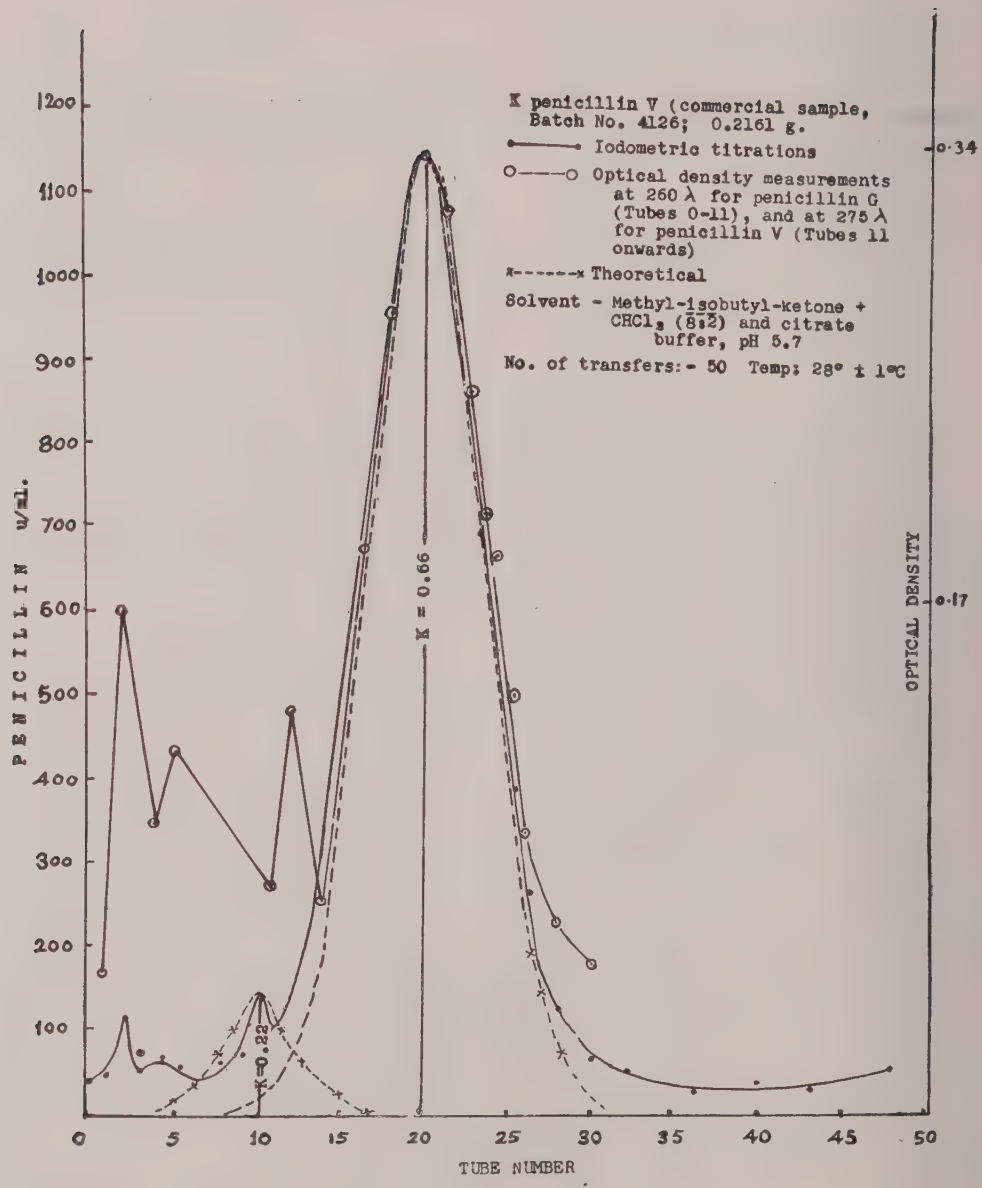


Fig. 1. Countercurrent Distribution Studies on Penicillin V.

The distribution pattern of penicillin V (Fig. 1) indicated three minor humps [tube Nos. 2, 4, 10 (K = 0 22)] in addition to a peak (tube No. 20 ; K = 0 66) both by the iodometric and the spectrophotometric methods of estimation. By the microbiological method, however, only one hump with maximum at tube No. 10 was observed in addition to the peak one. A parallel run with 100 mg. penicillin G (maximum at the 11th tube) and 100 mg. penicillin V (maximum at the 21st) indicated

that the penicillins could be easily separated from a mixture of both. A separate run with 10 mg. penicillin G and 90 mg. penicillin V, undertaken so as to overcome the mass effect[2], also showed the feasibility of easy separation. The humps due to penicillin G with maxima at tube Nos. 11 and 9 respectively in the last two experiments, indicated the possibility that the maximum at tube No. 10 of the distribution pattern of penicillin V might be due to penicillin G. This was further corroborated by the fact that the microbiological method of estimation demonstrated only one minor hump with maximum activity at tube No. 10 in addition to that due to penicillin V itself. It should be noted that the agreement of the theoretical curves for penicillin G and penicillin V, more particularly in the case of penicillin V, with the experimental ones is a perfect one. It was rather interesting to note that the distribution pattern showed a distinct hump with a maximum at tube No. 2. This showed a titre of 110 u/ml. (iodometric method) but was found to have negligible microbiological activity against *B. subtilis*. This may indicate the presence of a compound in the penicillin V sample susceptible to alkali decomposition and titrable with iodine but devoid of antibacterial activity.

Penicillin V, isolated in the form of free acid from the maximum tube (No. 20) and tubes around it, showed m.p. 127-28° (dec). Analysis : Found C, 55.05; H, 5.19 ; reported C, 54.85 ; H. 5.14%.

Calculations of the percentage of fractions, of penicillin V and penicillin G computed by counting the squares gave the following values : penicillin V 95.0 % ; penicillin G 2.9 %. The accuracy of the computation may be $\pm$ 1 %.

The distribution pattern of penicillin V in Sheehan's solvent phases (3) is almost identical with that described above.

Further investigations are in progress. Details will be presented elsewhere.

ACKNOWLEDGEMENTS

We wish to thank Dr. N. N. Chari, for the supply of penicillins, Mr. A. Thadathil, Dr. P. S. Borkar and Mr. A. M. Khan for their assistance in several estimations and Mr. A. V. Patankar, for carrying out microanalysis.

REFERENCES

1. Dasgupta, S. K., *et al.* (in preparation).
2. Craig, L. C., *et al. J. Biol. Chem.* **168**, 665 (1947). Bary, G. T., *et al. J. Biol. Chem.* **174**, 209 (1948). Craig, L. C., and Craig, D. Extraction and distribution. *In* Weissberger, A., *ed.* Technique of organic chemistry. New York, Interscience, 1950, v. 3, p. 171.
3. Behrens, O. K., *et al. J. Biol. Chem.* **175**, 771 (1948).
4. Bartels, C. R., and Dolliver, M. A. *J. Am. Chem. Soc.* **72**, 11 (1950).
5. Eisenlohr, H. *Chem. Ing. Tech.* **23**, 12 (1951).
6. Sheehan, J. C., and Henery-Logan, K. R. *J. Am. Chem. Soc.* **81**, 3093 (1959).
7. Parker, G., *et al. J. Pharm.* (*London*) **7**, 683 (1955).
8. Grove, D. C., and Randall, W. A. Assay methods of antibiotics. New York. M. D. Encyclopedia Inc., 1955.
9. Pharmacopeia of the United States of America Sixteenth revision (U.S.P. XVI) 1960, p. 496.
10. Weissberger, A., *ed.* Technique of organic chemistry, New York, Interscience, 1955, v. 7.

Spectrophotometric Determination of Procaine in Procaine Penicillin

N. NARASIMHACHARI & G. RAMANA RAO
Plant Laboratories, Hindustan Antibiotics Ltd., Pimpri, Near Poona

Various chemical and colorimetric methods are described in literature for the estimation of procaine.[1-6] A few of these can be employed for the determination of procaine content in procaine penicillin and some formulations containing them.

The maximum absorption of procaine at 290 mμ was used by St. John[3] for the determination of procaine in procaine penicillin. The absorption at 290mμ of a suitably diluted solution of procaine penicillin was compared with that of a solution of pure procaine hydrochloride or standard preparation of procaine penicillin. The results obtained by the author by this method were comparable with those obtained by pharmacopoeial methods although possibilities of interference by other compounds with the absorption at 290 mμ were indicated.

During the spectrophotometric investigation of a number of commercial preparations of penicillin, we had occasion to examine procaine penicillin samples. In these studies it was noted that the absorption at 290 mμ of different preparations varied widely, *a variation not found when other methods were used for procaine estimation.*

A pure sample of procaine penicillin G prepared from recrystallized potassium penicillin G, analysing for 40 % procaine content by the conventional pharmacopoeial method and the modified titrimetric method of Nayar *et al.*,[2] was taken as standard. The $E^{1\%}_{1\,cm}$ at 290 mμ with this sample, taken at different intervals is given in Table I.

TABLE I

STANDARD PROCAINE PENICILLIN

S. No.	$E^{1\%}_{1\,cm}$ at 290 mμ
1	288.0
2	285.0
3	289.0
4	285.0
5	289.4

% mean deviation = 0.63

Against this standard a number of procaine penicillin samples were examined for the procaine content employing the procedure described by St. John. The values obtained for some of the batches are given in Table II.

TABLE II

S. No.	$E^{1\%}_{1\,cm}$ at 290 mμ	Procaine content calc. from spectrophotometric reading	Procaine content by extraction method
1	288	40.00 (standard)	40.00
2	320	44.46	39.00
3	312	43.44	38.70
4	316	43.89	38.80
5	312	43.44	38.92
6	313	43.57	38.52
7	315	43.75	38.40
8	280	38.73	38.92
9	289	40.06	39.17
10	291	40.40	38.65
11	294.5	40.90	39.35
12	297	41.26	38.70
13	284	39.46	39.00
14	306	42.50	38.70
15	313	43.57	39.41
16	302	41.90	39.21
17	311	43.10	38.50
18	307	42.65	38.50
19	309	42.90	39.00
20	281	38.97	38.50

It will be seen from Table II, that the values of procaine content by spectrophotometry agree with those by the titrimetric method where the $E^{1\%}_{1\,cm}$ values at 290 mμ are close to that of standard procaine penicillin, whereas in cases of batches whose $E^{1\%}_{1\,cm}$ values are high, the procaine content estimated therefrom are higher than the theoretical value (40.15%) as also those obtained by the extraction method. All these batches under study did not however show any abnormalities in other properties such as potency and pH. These abnormally high values may be attributed to certain basic impurities associated with procaine hydrochloride or to a decomposition product of procaine having a very high specific absorbance at 290 mμ. It would, therefore, appear that the spectrophotometric method is inexpedient in the routine estimation of procaine content in commercial samples of procaine penicillin although it may serve to indicate the presence of interfering impurities in these preparations.

REFERENCES

1. British Pharmacopoeia, London, Pharmaceutical Press, 1958, p. 528.
2. Nayar, M. K., *et al J. Sci. Ind. Res.* (India) **17C**, 32 (1958).
3. St. John. C. V. *J. Am. Pharm. Assoc.* (*Sci. Ed.*) **37**, 343 (1948).
4. Pharmacopoeia of India, Delhi, Manager of Publications, 1955, p. 497.
5. Ashley, M. G., and Lees, J.F.*J. Pharm.* (*Lond.*) **6**, 50 (1954).
6. Srinivasan, K. R. *Analyst* **75**, 76 (1950).

5. Farmitalia*

S.A. Farmaceutici Italia
(Gruppo Montecatini)
MILANO

The Farmitalia, an associate company of the Montecatini Group, first started manufacture of chemicals and pharmaceuticals with the preparation of chemical products for pharmaceutical use, and of medical specialities. In 1937, the policy of keeping production abreast of the requirements of modern therapy, led to the inception of a Research Centre to work in the field of pharmacology, chemotherapy and pharmaceutical chemistry.

From initial research on synthetic antimalarial drugs, work was continued on the arsenical, bismuth and gold chemo-drugs, followed by sulfa drugs and vitamins, until the present stage of activities was reached. Today a new plant handles the large-scale production of a wide range of synthetic and fermented antibiotics and related specialities for human and veterinary use. One of the most important of these departments is that in which the complete manufacturing cycle of corticosteroid hormones is gone through. Numerous other intermediate and basic products for the pharmaceutical and perfumery industries are also produced in this plant.

Farmitalia today comprises the following research and production units :

Laboratories for chemical, microbiological-chemotherapeutical and pharmacological research, located at Milan.

Veterinary Research Laboratories at Nerviano (Milan).

Manufacturing plant for chemico-pharmaceutical products (organic and inorganic), synthetic and fermented antibiotics and steroids, at Settimo Torinese (Turin).

Manufacturing plant for the preparation and packaging of medical specialities, also at Milan.

Chemical, Microbiological-chemotherapeutical, Pharmacological Research Organization

The Farmitalia Research Laboratories, covering an area of 8,000 sq. metres, of which 4,700 are built up, comprise of three main sections — chemical, microbiological-chemotherapeutical and pharmacological. These laboratories equipped with the latest types of scientific equipment and employing 300 people, of whom over 100 are university professors, graduates and specialists in various fields, are designed to meet the requirements of the most progressive techniques in experimental science. A well stocked library contains the major works of reference and most important scientific journals from all over the world. The laboratories thus offer full scope for the study of all aspects of chemotherapy.

Veterinary and Zootechnic Research Laboratories

These well equipped and up-to-date laboratories with a staff of about 80 are located at the new Cattle Breeding and Veterinary Test Centre, at Nerviano (Milan) on an area of 38 hectares, for the study and control of :

(a) Drugs for the treatment of infectious and infestatious cattle diseases ;

(b) growth factors and utilization factors in cattle feeding.

Settimo Torinese Plant

The Settimo Torinese Plant is the basic industrial core of Farmitalia, and is con-

* *Courtesy :* Farmitalia, Milan, Italy.

sidered as one of the major pharmaceutical manufacturing centres in Europe today. This unit manufactures both the pharmaceutical products which are further processed into specialities, and those supplied to other domestic and foreign industries. The plant covering 150,000 sq. metres, of which 60,000 are built up, has features, facilities and equipment typical of modern progressive industrial pharmaceutical enterprises. The staff numbers 1,300 of which 200 are university graduates. The most important of the range of compounds produced in this plant are :

Fermented antibiotics : Penicillins in their different forms (G, V, DBED, etc.) dihydrostreptomycin, chlortetracycline, tetracycline, spiramycin, cycloserine, aminosidina, etruscomicina.

Synthetic antibiotics : Chloramphenicol (Farmicetina) and its salts.

Steroids : (Proteic anabolizing, progestative, corticosteroid). *Sulfa-drugs.*

Anti-tubercular (chemotherapeutical) agents : PAS and its salts, isoniazide, etc.

Liver extracts.

Choleretics : Cynarine.

Barbiturics.

Analgesics, antipyretics and antirheumatic drugs : Salicylic and pyrazol drugs, etc.

Spasmolytics.

Anesthetics.

Tranquilizers.

Miscellaneous organic and inorganic products : Guaiacol and derivatives, piperazine and its salts, theophylline, synthetic antihistamines, curare derivatives, mercury salts, glycero-phosphates, phenophthalein, etc.

Products and specialities for veterinary use.

Cattle food integrators : Feed supplements.

Basic products for the perfumery and confectionary industries : Vanillin, ethylvanillin, coumarine and various esters.

Milan Plant

The production cycle is completed in this plant with the preparation of Farmitalia's speciality medicines.

Built in 1952, this plant covers an area of 70,000 sq. metres, of which 25,000 are floor surface. The main part consists of three factory buildings, each with five stories and basement, ranged at right angles to a fourth lower building which forms sort of a back to the comb layout. The production flow is designed on the gravity system with carefully planned routes. On the last floor of one of the larger buildings, is installed the unit for sterilizing ampoules bottles, stoppers etc. Next, coming down, are the equipment for dividing up and packaging antibiotics, freeze-drying and sterile filling of injectables, powders and solutions.

The Laboratories for making chemical and sterility tests, the pharmaceutical methods laboratory and the ampoule, injectable solutions, tablets, pills, capsules, "cachets", suppositories and syrups departments occupy the other buildings of the plant. In these laboratories the identity and characteristics of the pharmaceutical products from the Settimo Torinese plant and of other raw materials and purchased materials and products are thoroughly checked and attested.

The Biological Test Laboratories carefully test each drug for toxicity, which must be within the standard limits and must have undergone no change during processing due to impurities (which are sometimes not detected by chemical or

chemicophysical methods), or bacterial contaminants (pyrogenic substances) which may not be evidenced by microbiological methods. Other specialised laboratories carry out sterility tests on medicines for parenteral use. Lastly, the pharmaceutical technology laboratory carries out a series of physical, chemical, physico-chemical and microscopical investigations to ascertain that the active components of the medicine are in the best possible condition to be of the greatest therapeutical efficacy, and that the method of preparation ensures its perfect preservation.

Farmitalia markets its drugs through a wide organization of agents and representatives in over 65 countries. The complex and elaborate system of control ensures the purity, proper make up and therapeutic efficacy of Farmitalia's products the world over.

Edited by Dr. M. J. Thirumalachar and Published by Shantikumar T. Raja, B.A., LL.B. Bar-at-Law, Hindustan Antibiotics Ltd., Pimpri (near Poona). Printed by Rev. Theodore A. Pereira at the Examiner Press, Bombay—1.